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T cell activation by ethanol: A possible mechanism for immunosuppresssion.

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T cell activation by ethanol: A possible mechanism for immunosuppresssion.

by

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Dedication

To my wife Malika for understanding, encouraging and supporting me through this process and for giving me our beautiful daughter Khadija.

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T cell activation by ethanol: A possible mechanism for immunosuppresssion.

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Alcohol abuse has been commonly associated with enhanced susceptibility to pathogens. Studies on the effects of ethanol on the immune system are complicated by a lack of consensus on whether ethanol activates, inhibits or has no effect on immune cells. We present data showing that acute exposure of T cells to ethanol elicits responses that broadly parallel responses seen in normally stimulated T cells such as the formation of the immune synapse, polarization of the microtubule organizing center (MTOC) to the synapse and tyrosine phosphorylation of signaling proteins as seen when the T cell Receptor (TcR) engages antigen-MHC. However, incomplete activation of the T cell signaling program leads to unresponsive or anergic T cells. Our data suggests the hypothesis that ethanol can activate T cells in a manner that leads to anergy.

We have found that ethanol triggers calcium signaling and this has provided one of the primary tools for analyzing the effects of ethanol on T cells. Ethanol induced calcium transients are dose-dependent and are comparable to those triggered by low doses of anti-TcR antibody. This is important because it allows us to compare ethanol-

dependent signaling to that normally triggered through stimulation of the T cell receptors. Analysis of the calcium signaling pathway indicates that ethanol-stimulated calcium transients depend on calcium entry and are likely due to opening of CRAC type calcium channels. The observed calcium transients go a long way towards explaining how ethanol may stimulate T cells and provides a mechanism for immune suppression through the observed translocation of NF-AT in ethanol pulsed cells. The translocation of NF-AT is particularly important because of reports that it plays a crucial role in triggering anergy and immunosuppression. Taken together, these data can help explain how ethanol can both activate T cells and cause immunosuppression.

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Chapter 1: Background and Significance

INTRODUCTION

Alcohol over consumption is the third leading cause of death in the United States after tobacco intake and poor diet and exercise [1]. Abuse of alcohol leads to serious medical problems for the individual and negatively impacts nearly every system of the body[2, 3]. Alcohol abuse also leads to a host of other problems that add up to exact a staggering economic cost. According to one estimate, alcohol abuse cost the US economy 186 billion dollars in 1998[4]. Almost half of that figure (88 billion) is attributed to lost productivity due to alcohol related illness, while 19 billion dollars were diverted to the medical treatment of alcohol related conditions. Automobile accidents caused by DWI (Driving While Intoxicated) (in 2005, 16,885 or 39% of all traffic fatalities, were alcohol related[5, 6]) as well as binge drinking deaths (1,400 fatalities a year according to one estimate [7]) are widely publicized.

Among the medical problems of alcohol over consumption are damage to the developing fetus resulting in fetal alcohol syndrome[8], impaired ability to recover from injury, immunosuppression resulting in increased susceptibility to infections and cancer[9, 10] brain damage[11], liver disease[12], and heart disease in the form of Alcoholic Myocardiopathy[13]. Ethanol over consumption also leads to increased resistance to insulin and impairs insulin-mediated glucose uptake [14-16]. It is interesting to note that women are more vulnerable to organ damage as compared to men for the same consumption level of alcohol [17, 18].

While over consumption of alcohol is associated with many medical problems, alcohol in moderation may also have some health benefits. Beer and wine were used for medicinal purposes in Sumeria as early as 2000 B.C. and a Chinese imperial edict dating

back to 1116 B.C., stated that the use of alcohol in moderation was prescribed by heaven [19]. More recently, there have been reports of moderate alcohol intake increasing insulin sensitivity, thus benefiting diabetics [5, 6, 20]. Other advantageous effects of moderate ethanol intake include cardio protective effects[21] in part through the elevation of High Density Lipoproteins (HDL) [22], inhibition of platelet aggregation [23] as well as improved endothelial cell function by promoting the secretion of Nitric Oxide (NO) resulting in vasodilation [24]. Alcohol is also seen to have protective effects against renal ischemia/reperfusion injury[25]. One study has described the use of high concentration ethanol as a local adjuvant for giant cell tumors in the bone[26] while another has even suggested a role for ethanol as a "gastric disinfectant" [27].

THE IMMUNE SYSTEM

Our particular focus concerns the effects of alcohol on the immune system and a brief overview of the immune system is given here. The immune system is broadly divided into two main branches: innate and acquired immunity[28]. Innate immunity generally involves responses to specific pathogens or specific aberrations that are commonly encountered by the organism. It is in place before the body comes in contact with a pathogen for the first time and immediately responds to predefined markers on bacterial or other pathogens that enter the body. Examples of innate immunity include phagocytic cells and their secreted signaling molecules known as cytokines, natural-killer (NK) cells, and specialized proteins known as complement. The NK cells are cytotxic lymphocytes that eliminate tumor as well as infected cells in the body[29]. In this regard, NK cell function broadly parallels that of cytotoxic T cells, which are involved in acquired immunity. NK cells however do not express the T cell receptor, or other markers (CD3, B cell receptor) that are found on T and B cells. These cells survey for cells lacking appropriate MHC[30].

Phagocytes consist of two cell types:

1. Neutrophils destroy invading microbes by ingesting them into a phagosome into which reactive oxygen species (ROS) and proteolytic enzymes are released destroying the pathogen. These cells primarily destroy invading bacteria.
2. Monocytes circulate in the blood. Once they migrate from the bloodstream to other tissues they become known as macrophages. These cells also ingest microorganisms and display pathogen proteins in conjunction with the Major Histocompatibility Complex (MHC) to activate other cells in the immune system.

Innate immunity is involved in the first phase of the immune response; namely the inflammatory reaction which protects the body from immediate effects of an infection. This involves the destruction of pathogens by phagocytes and the release of cytokines that recruit more immune cells to the site of an infection (such as a cut) as well as other molecules (e.g. ROS) that assist in the killing the pathogen[31].

Acquired immunity is activated once the body is exposed to a pathogen. This system is more versatile because it can respond to almost any pathogen and yet will be exquisitely specific to that particular pathogen. T and B lymphocytes together with antigen presenting cells (dendritic cells, macrophages) are the most important cell types involved in acquired immunity[32]. B and T cells generally circulate in the blood stream or reside in the lymphoid tissue (e.g. spleen, lymph nodes) but they can also infiltrate into tissues. The immune response is largely orchestrated by helper T cells through their secretion of different cytokines that direct the activation of cytotoxic T cells (Th1 response) or B cell responses (Th2 response) or both. The Th1 and Th2 subgroups are based on the pattern of cytokine expression[33];

a. Th1 cells produce interleukin (IL)-2, interferon (IFN)-γ, and Tumor Necrosis Factor (TNF)-β. These cells are involved primarily in promoting cell mediated immunity.
b. Th2 cells produce IL4, IL5, IL6 and IL13 and are involved primarily in promoting humoral immunity by stimulating B cells to produce immunoglobulins (Ig).

The Th subsets differentiate from the same precursor cells known as naïve CD4+ T lymphocyte or Th0. The Mature Th0 cells produce IL-2 upon encountering antigen and are stimulated to differentiate by other cytokines. In the case of bacterial infection or low antigen concentration, macrophages release IL-12 stimulated by microbial endotoxin, components of viruses and bacteria, which in turn stimulates differentiation of Th0 cells into Th1 cells[33].This happens through the IL-12 mediated activation of transcription factors such as Stat 1, Stat 3 and Stat 4. IFN-γ inhibits differentiation into Th2 subsets and also helps in the differentiation of Th1 cells by stimulating macrophages to release more IL-12.

In the case of attack by parasites, IL-4 is released by Th0 cells which in turn stimulates the secretion of more IL-4 through the activation of the transcription factor Stat 6[34, 35]. The initial transcription of IL-4 is thought to be mediated by the transcription factor GATA-3[36]. Another transcription factor Stat 5, has been reported to be activated by GATA-3 which in turn also promotes the transcription of the IL-4 gene[37]. IL-4 in turn inhibits differentiation into Th1 subsets[33]. GATA3 expression has also been implicated in inhibiting Th1 differentiation[38].

Th1 dominant immunity is associated with the inflammatory response as well as cellular immunity (associated with delayed type hypersensitivity)[39]. The Th1 cytokines, IFN-γ and TNF-β recruit and activate inflammatory leukocytes. In mice, IFN-γ

not only activates macrophages to phagocytose and destroy microbes, but also stimulates B cells to secrete immunoglobulin G (IgG) which has a high affinity for the Fcγ receptor and complement proteins[33]. The Fcγ receptor binds to the Fc fragment of the IgG molecule and is present on the surfaces of cells such as macrophages, where upon binding it stimulates the phagocytosis of opsonized (coated with antibody) bacterial particles[40]. IFN-γ along with IL-2 leads to the activation of CD8+ T cells.

Th2 dominant immune response involves humoral immunity (associated with immediate hypersensitivity or allergic reactions)[33]. IL-4, one of the Th2 associated cytokines, induces B cells to produce IgE[41] which binds to Fc receptors on the surface of mast cells and basophils "sensitizing" them. Upon subsequent exposure to the same allergen, the cells undergo de-granulation releasing histamine, leukotriene and prostaglandins that act on surrounding tissue[42].

As mentioned above, the development of Th0 cells into Th1 or Th2 subsets is tightly controlled with the development of one subset leading to the inhibition of the other. It is thought that low dose of antigen and low dose infections promote the development of Th1 while high doses promote Th2 development[33]. Upon encountering low dose of antigen, macrophages and dendritic cells act as antigen presenting cells and release IL-12 which promotes Th1 differentiation. On the other hand, high levels of antigen tend to stimulate T cells repeatedly leading to IL-4 production or inducing a state of tolerance which favors shutting down Th1 differentiation. It has been observed that inducing anergy using peptide in human T cell clones isolated from peripheral blood mononuclear cells (PBMCs) leads to increased IL-4 production[43]. Interestingly, treating dendritic cells with ethanol leads to reduced IL-12 production and naïve CD4+ T cells primed with such dendritic cells show hypo-responsiveness when subsequently stimulated with untreated dendritic cells[44].

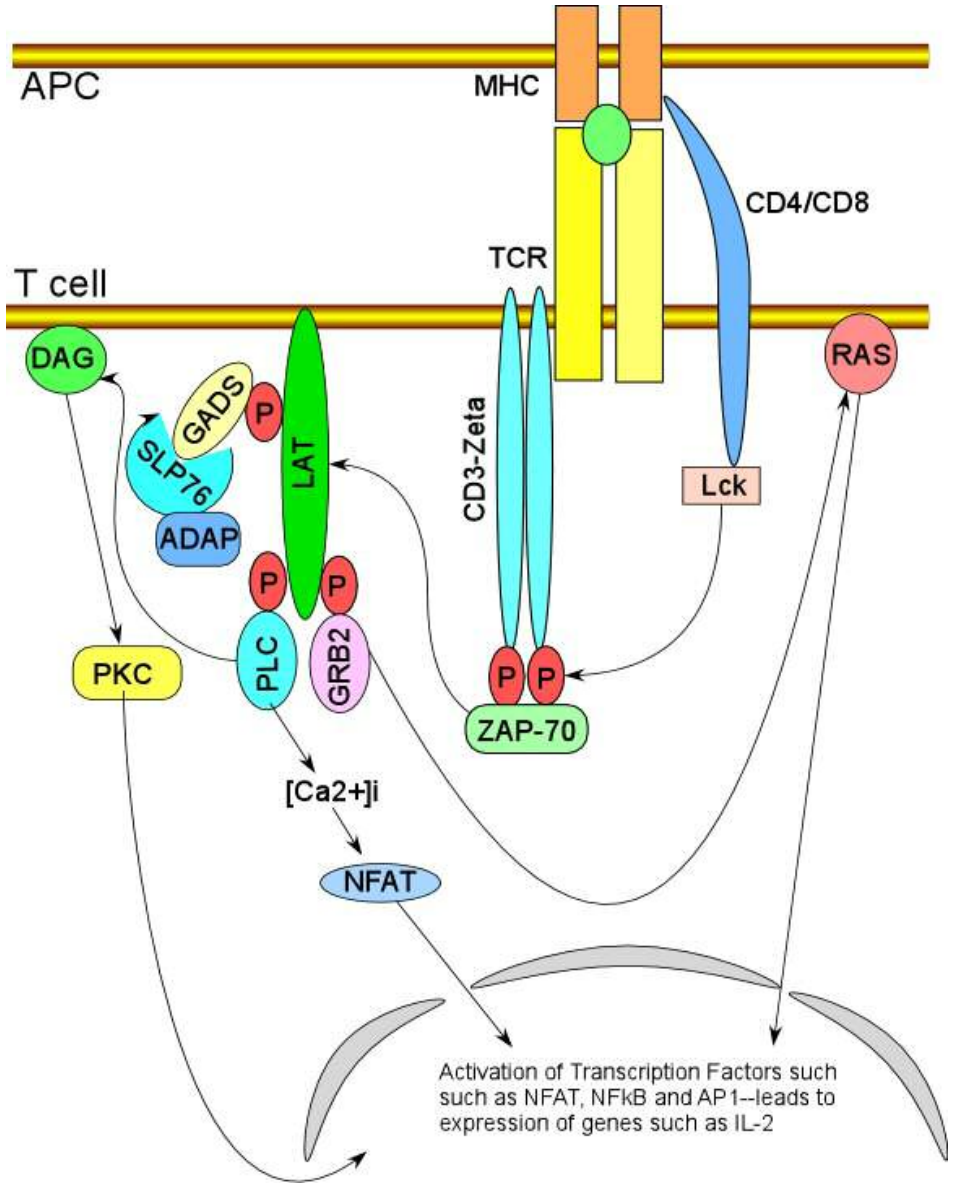

Figure 1.1: An over view of T cell signaling during activation.

Binding of T cell receptor (TCR) to MHC-bound antigen leads to the clustering of the TCR together with CD4 or CD8 and Lck, leading to phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMS) on the TCR CD3-ζ chain. These phosphorylated ITAMS serve as a docking site for the protein tyrosine kinase ZAP-70 which phosphorylates a number of downstream signaling proteins including LAT. Phosphorylated LAT serves as a docking site for signaling proteins such as PLC-γ, GRB-2 and GADS that convey the activation signal downstream resulting in the expression of genes such as IL-2.

INTRODUCTION TO T CELL SIGNALING:

Activation of T cells is initiated by the T cell receptor (TcR). The TcR is actually a signaling complex that is composed of different peptides such as the α and β chains, a signaling subunit called the CD3 complex (which is composed of the γ, δ and ε chains) and the ζ chain [45]. Binding between the T cell receptor (TcR) and the foreign peptide presented on an MHC molecule results in TcR clustering. This results in a wave of early protein tyrosine kinase (PTK) activity mediated by the Src family kinases $P56^{lck}$ and $P59^{fyn}$. The exact mechanism of Lck and Fyn activation is unclear, but appears to involve the removal of the inhibitory C-terminal tyrosine phosphate residues by CD45 tyrosine phosphatase. In activating Lck, CD45 forms multimeric complexes with CD4 or CD8 which is physically associated with Lck[46]. In the case of Fyn activation, the mechanism of CD45 interaction with Fyn is unclear. The activated Src kinases then phosphorylate paired tyrosines on the cytoplasmic tails of the TcRζ chains. These paired tyrosines are known as the immunoreceptor tyrosine-based activation motifs or ITAMS. The activated Src kinases also regulate the function of other kinases such as the Tec family of kinases and more importantly that of ZAP-70.

ZAP-70 (ζ-chain associated protein kinase of size 70 KDa) binds to the ITAMS through its paired SH2 domains and is then phosphorylated by Lck (Figure 1.1). This in turn, causes the binding of the Lck SH2 domain to ZAP-70. This interaction is critical for sustained ITAM phosphorylation, and leads to the recruitment of additional ZAP-70 molecules as well as other Lck substrates such as the Tec kinase Itk[47]. The recruitment of ZAP-70 to the ITAMs in turn leads to the phosphorylation of LAT (Linker for activation of T cells) [48] an adaptor molecule which in turn binds to other signaling proteins such as Phospholipase C gamma (PLCγ). LAT phosphorylation and the consequent scaffold assembly relay the activation signal to downstream signaling

proteins such as SLP 76 (SH2 domain-containing leukocyte protein of 76KDa), ADAP and Gads to organize the architecture of the contact site between the T cell and its target APC [47]. Phosphorylated LAT also binds to another adaptor known as the growth factor receptor bound protein (Grb-2) which binds son of sevenless (Sos) leading to the activation of the Ras pathway [48]. This in concert with other signaling cascades namely JNK, NFAT and NFκB lead to the transcription of genes that promote survival and proliferation.

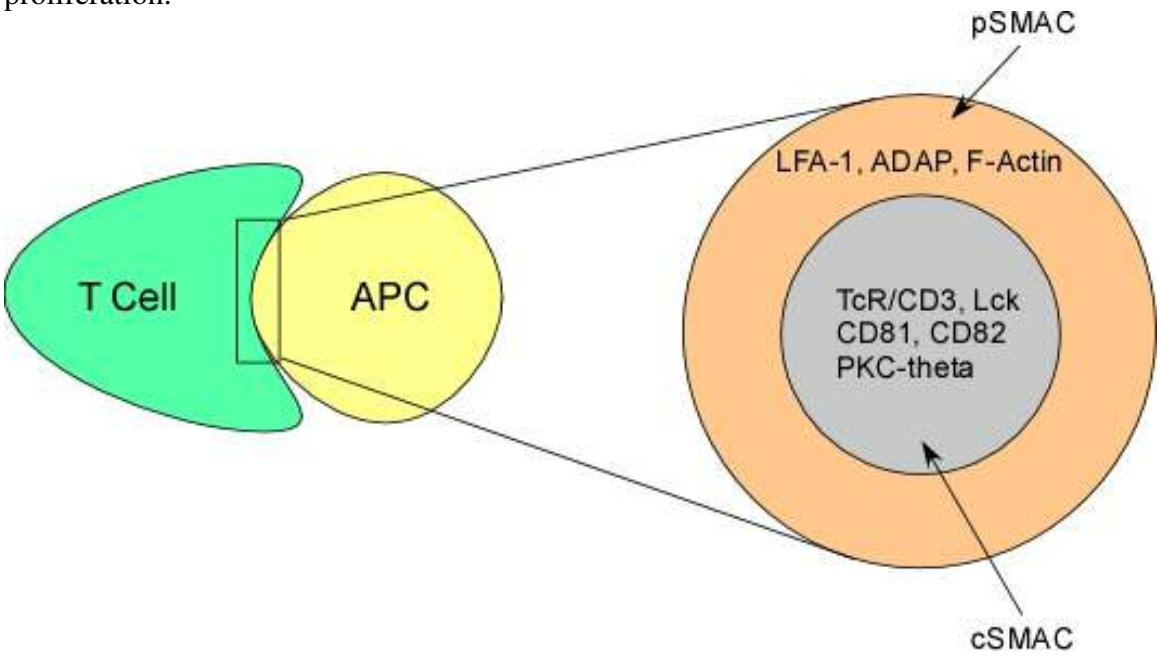

Figure 1.2 Cross section of the mature immune synapse.

This cross section shows that the T cell receptor along with signaling kinases are concentrated in the central SMAC while the periphery is composed of rings of adhesion molecules such as LFA-1 and ADAP as well as skeletal proteins such as Actin.

Besides activating PLCγ by recruiting a Tec kinase, SLP-76 also recruits ADAP and Vav, a GTP exchange factor for Rho GTPases [49]. Vav plays a role in regulating actin assembly[45]. These events ultimately lead to the formation of a specialized contact

area known as the immunological synapse (IS). The IS is traditionally divided up into two zones known as supramolecular activation clusters (SMACS)[50] (Figure 1.2). The central SMAC (cSMAC) contains TcR, Lck, Fyn, CD4 or CD8, the tetraspan molecules CD81 and CD82 and PKCθ among many others[45]. The outer or peripheral SMAC (pSMAC) is a ring like structure, which contains F-actin, ADAP and LFA-1. The formation of the IS sets the stage for the translocation of the microtubule organizing center (MTOC) up to the IS followed by the accumulation of secretory vesicles and directed secretion of effector molecules [51].

ROLE OF CALCIUM IN T CELL ACTIVATION

As mentioned previously, activated LAT binds PLCγ which is phosphorylated by the tec kinase Itk which itself is bound to LAT via SLP-76 and GADS [45, 52]. Activated PLCγ in turn cleaves phosphatidyl inositol 4,5 bisphosphate (PIP_2) into diacyl glycerol (DAG) and inositol trisphosphate (IP_3)[47]. IP_3 in turn releases calcium from stores in the endoplasmic reticulum (ER)[53]. This depletion of calcium stores leads to a prolonged influx of calcium through store operated channels (SOCs)[54]. There are several possible types of SOCs including calcium release-activated or CRAC channels [53]. Other channels such as the transient receptor potential (TRP) channels may also be involved in calcium flux. The function of TRP channels in T lymphocyte activation remains unknown [55]. A recent study has suggested that some TRP channels may serve as a channel for bivalent cations including calcium [56]. However, some such as Richard Lewis have indicated that CRAC channels are the sole entry way for calcium influx following activation[57].

CRAC channels consist of two components, one in the ER (STIM-1) that serves as a sensor for store depletion and another in the plasma membrane (Orai-1) that forms the actual channel[58]. Recent studies have shown that upon the depletion of calcium

stores in the ER, STIM-1 moves from locations throughout the ER to discrete regions within the ER next to the plasma membrane. At the same time Orai-1, gathers at distinct sites on the plasma membrane directly opposite the sites where STIM-1 has clustered resulting in localized CRAC channel opening and elevation in $[Ca^{2+}]_i$ (intracellular calcium) [58].

Calcium influx through the CRAC channels appears to be responsible for the bulk of the calcium increase seen at the onset of T cell activation and is also required for the prolonged oscillations in $[Ca^{2+}]_i$ that are required for a successful T cell response[53] . The frequency of these $[Ca^{2+}]_i$ oscillations plays a central role in the activation of T cells by controlling the genes that will be expressed [54]. For example, the nuclear factor for activation of T cells (NFAT) activation requires $[Ca^{2+}]_i$ oscillations with a period of less than six minutes, while NFκB becomes activated even with time periods as long as 30 minutes [54]. Together with other transcription factors such as c-Fos and c-Jun (AP-1), NFAT and NFκB result in the transcription of genes that promote T cell survival and proliferation [45].

T CELL ANERGY

Anergy is a tolerance mechanism where T-cells remain in a state of inactivity following antigen presentation, but do not undergo apoptosis or cell death [59]. It is defined as the inability of a T cell or T cell clones to re-stimulate with an appropriate antigen and APC [60]. It is thought that anergy arises when the T cell does not receive a complete stimulus. It has long been known that T cells require accessory stimuli or co-stimulation along with signaling through the TcR. The major co-stimulation pathways involves binding of CD-28 on the T cell with members of the B7 family of ligands on the APC[45].

CD-28 costimulation is important in the activation of transcription factors that transcribe genes such as IL-2 which in turn lead to the survival and proliferation of T cells. Activation of transcription factors such as NFκB depends on signals both from TcR stimulation as well as co-stimulatory signals from the ligation of CD-28 [61]. NFκB is kept sequestered in the cytoplasm through its association with IκB which exists in two forms IκB-α and IκB-β. Diacylglycerol, a product of PIP2 cleavage by PLCγ activates PKCθ which in turn phosphorylates IKK, a kinase for the inhibitory kappa B subunit (IκB) [45]. CD-28 costimulation plays an important role in the recruitment of PKCθ to the immune synapse where it is activated[45] and has been reported to lead to the down regulation both the α and β isoforms of IκB[62]. Upon T cell activation, IκB is phosphorylated and targeted for ubiquitination and destruction [63]. This frees NFκB which translocates to the nucleus and resides there until enough IκB is synthesized to bind and hold NFκB in the cytosol[54].

A key gene target for activated NFκB is the CD-28 response element (CD-28-RE) in the IL-2 promoter [45]. This is a combinatorial response element which requires c-Fos and c-Jun (AP-1) as well as NFκB for full activity. Jun is activated through the JNK pathway which is also induced by co-stimulation through CD-28[64]. Another target for NFκB is the Bcl-$_{XL}$ promoter. The Bcl-$_{XL}$ gene promotes cellular survival in response to co-stimulation [65].

In the absence of co-stimulation, the cells would not be able to transcribe genes that promote survival and proliferation. Anergy, therefore, arises when T cells are challenged without co-stimulation of the CD-28 receptor[66]. In addition to this "signal-1 alone" model for inducing anergy, other mechanisms have also been described. Altered peptide ligands (APL) can also induce anergy [67]. APLs are altered in such a way that they still have the ability to bind the TcR but are unable to induce full activation of the T

cell. Even in the presence of co stimulation, APL binding to the TcR induces a state of hypo-responsiveness.

NFAT has gained a lot of importance in the thinking about the induction of anergy since it does not require co-stimulation for activation. NFAT is activated by calcineurin, a calcium/calmodulin serine phosphatase that dephosphorylates multiple residues on the protein to expose the nuclear localization signal [68]. This de-phosphorylation is countered by a robust phosphorylation through a number of inducible/constitutive kinases so that once $[Ca^{2+}]_i$ levels fall below threshold, NFAT is re-phosphorylated so as to expose its nuclear export signal leading to its transport out of the nucleus [54].

If NFAT is activated along with NFκB and AP-1, the result is transcription of effector genes such as IL-2. However, in the absence of other transcription factors, activated NFAT enters an alternate transcription program where it transcribes E3 ligases such as Gene Related to Anergy in Lymphocytes (GRAIL), Cbl-b and Itch [69, 70]. Sustained calcium signaling also increases the expression of Tsg101, the receptor involved in sorting mono-ubiquitinated proteins to the lysosomal degradation pathway. Itch targets PKCθ and PLCγ for mono-ubiquitination and lysosomal degradation[71].

Induction of anergy has therapeutic value in preventing rejection of transplanted tissue/organs. Administration of an anti TcR antibody (OKT3) is often the last resort for preventing graft rejection [72].

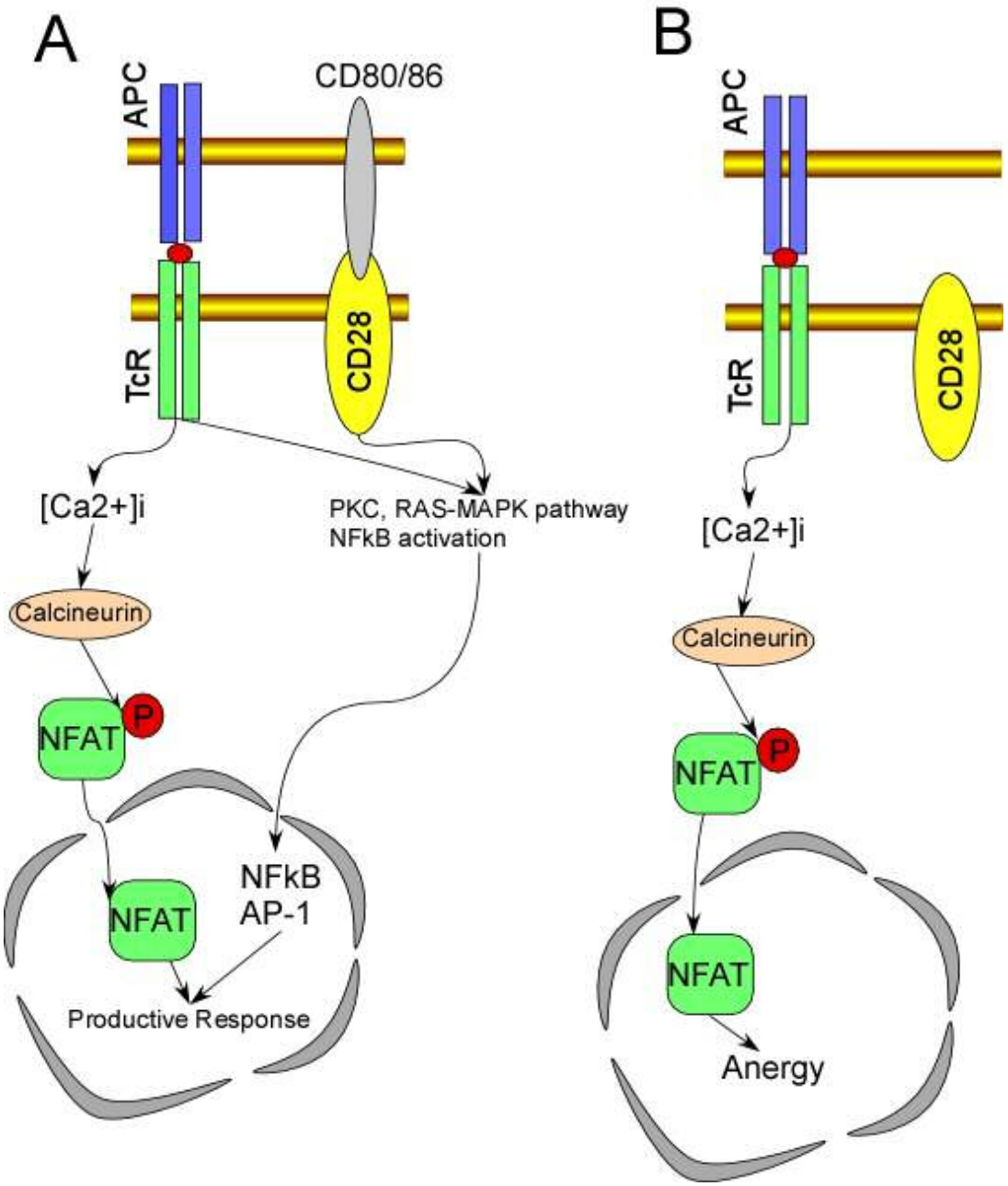

Figure 1.3: NFAT directed gene expression; T cell activation versus anergy.

A) Signals transmitted by the engagement of the TcR as well as CD28 result in the activation of many different transcription factors. This leads to the transcription of genes that promote proliferation and survival. B) When TcR engagement occurs in the absence of co-stimulation, the elevation in $[Ca^{2+}]_i$ results in the activation of NFAT alone which in the absence of other transcription factors transcribes genes that result in anergy.

ETHANOL AND THE IMMUNE SYSTEM

Our interest is in how alcohol intake affects the immune system particularly with reference to T cell activation and function. Alcohol has been widely recognized as an immunosuppressant. As early as 1785, Benjamin Rush, the first Surgeon General of the United States reported that alcohol users are predisposed to more frequent and to more severe infections, particularly of the respiratory tract[73]. Among the reported examples of immunosuppression are enhanced susceptibility to opportunistic microbes [74], accelerated progression of diseases such as Hepatitis-C [75] and increased cancer [76].

However, despite the consensus on the immunosuppressive effect of alcohol, research into the specific effects of alcohol on T cells have resulted in disparate conclusions. Some studies suggest that ethanol inhibits T cell functions[77, 78], whereas other studies show that ethanol activates T cells[79] or has no effect[80, 81]. Recent studies suggest that acute and chronic alcohol intake tends to activate certain aspects of the immune system while inhibiting others with the result that ethanol abuse leads to both immunosuppression as well as tissue damage. For example, acute ethanol intoxication reduces the expression of inflammatory cytokines TNF-α and IL-6[82], as well as super oxide ion and nitric oxide—critical components of pulmonary host defense thus rendering alcoholics more susceptible to pulmonary and other infections [83].

On the other hand, rats fed ethanol chronically show increased expression of the integrin CD-18 in neutrophils[84]. This allows the cells to adhere to and migrate through cells lining blood vessels (endothelial cells) to the site of infection and destroy pathogens. Moreover, ethanol induces Kupffer cells (macrophages residing in the liver) to release cytokines such as IL-8 that results in enhanced chemotaxis of neutrophils[85]. Ethanol also causes increased expression of the ligand for CD-18 (ICAM-1) in hepatocytes[86]

thus aiding in inducing hepatic injury and the onset of Alcoholic Hepatitis. Conversely, neutrophils from chronically alcoholic human subjects show decreased chemotaxis which may in turn contribute to the susceptibility of alcoholics to infection[31].

NK cells are involved in the destruction of infected and tumor cells that are not recognized by T and B lymphocytes due to low levels or absence of MHC. Acute alcohol intoxication in rats suppressed NK cell activity and reduced the animals' ability to prevent metastases in an NK-sensitive tumor [87]. Recently, this suppression in NK cell activity has been linked to reduced expression of cytokines that promote the inflammatory response[88].

In the case of B-cells, it has been observed using mouse feeding models that chronic exposure to ethanol leads to reduction in the number of immature B cells in the bone marrow, while withdrawal of alcohol leads to lower number of mature B cells and an even greater loss of immature B cells in the bone marrow [89]. With respect to antibody production, earlier studies suggest that ethanol exposure tends to lower antigen stimulated antibody production[90, 91] while more recent work shows that chronic alcoholics tend to have elevated serum antibody levels [31].

ETHANOL AND T CELL ACTIVATION

A number of studies report that ethanol intake tends to suppress T cell function. Impaired T cell proliferation has been observed in both long term (30 days)[92] and short term (4—48 hours)[93] ethanol exposure. Acute ethanol intake is also reported to promote T cell apoptosis[94]. One study suggests that acute ethanol exposure interferes in CD-18 mediated adhesions and also diminishes the production of IL-2 [95]. Other studies report impaired function of antigen presenting cells (APCs) as the reason for impaired T cell function[44, 96].

The link between alcohol consumption and aggravation of AIDS has been particularly interesting and important [97-99]. Recent studies have shown that acute alcohol ingestion increases the speed of transformation from HIV infection to illness[100]. Chronic alcohol consumption has also been shown to accentuate the severity of murine-AIDS (MAIDS) [101]. Perhaps the most convincing link between AIDS and alcohol consumption comes from the work of Bagby *et. al.* [102] demonstrating that ethanol administration to rhesus macaques displayed a 60-fold higher SIV viral RNA load than the control group given an iso-caloric sucrose solution. Alcohol consumption has also been suggested as being responsible for CD4+ lymphocyte death, thus aggravating AIDS[103]. Ethanol was also found in Jurkat helper T lymphocytes to induce the inflammatory cytokine Tissue Necrosis Factor-Alpha (TNF-α) which in turn promotes the HIV-1-LTR directed transcription as well as the translocation of NFκB into the nucleus thus facilitating HIV replication[104].

One study that clearly demonstrated that ethanol inhibits T cell activation was conducted by Saad *et. al* [105]. Ethanol severely impaired the ability of mice to overcome infection with *Listeria monocytogenes,* an immune response mediated entirely by cytotoxic T lymphocytes (CTLs). *Listeria monocytogenes* is an intracellular bacteria that has a predilection for the liver and is used to study cell mediated immunity[106]. Owing to its intracellular nature, elimination of the bacteria depends on destruction of the infected cells. In this study, C57Bl/6 mice were given a diet containing 7% (v/v) ethanol (~1.2M) for 1 week before being infected with the bacteria. 5 days after the infection, mice that had been fed ethanol had considerably more liver damage and cleared 1000 times less bacteria than their non alcoholic counterparts. Even in mice with preexisting immunity to *L. monocytogenes* alcoholic mice had 100 times more bacteria in their livers as compared to non-alcoholic mice. The study found fewer T cells in the foci of

infections in liver sections from alcoholic animals even though there were larger areas of hepatic necrosis with a greater abundance of inflammatory cells when compared to liver sections from non-alcoholic animals.

By contrast, some studies show that ethanol activates T cells. Santos-Perez *et. al.*[107] have shown that ethanol exposure up-regulates the expression of the IL-2 receptor CD-25, the integrin LFA-1 and the production of cytokines such as IL-2, TNF-α and interferon-gamma (IFN-γ). Given that CD-25 up regulation is a normal outcome of T cell stimulation by targets and that LFA-1 up regulation requires inside out signaling, this data suggests that ethanol can cause normal activation of T cells, at least to some extent.

These studies reporting both the activation and inhibition of T cells by ethanol present something of a paradox. In the studies detailed in this dissertation, we present data that goes a long way towards resolving this paradox. Using markers of T cell activation such as the formation of the immune synapse, tyrosine phosphorylation as well as calcium flux we show that acute exposure to ethanol does activates T cells. However, when exposure to alcohol is prolonged (3—4 days) T cells lose their response to TcR stimulation in a manner that closely parallels that seen when the TcR is chronically ligated using antibody. Thus our studies support the hypothesis that T cell activation by ethanol triggers a loss in responsiveness similar to anergy.

Chapter 2: Acute Ethanol Activation of T cells

INTRODUCTION

Chronic ethanol over-consumption is associated with increased susceptibility to infections and cancer. Previous studies have suggested that exposure to ethanol both activates[79] and suppresses[108] the immune system and in particular T cell-mediated immunity. However, few reports have studied the signaling events that lead to ethanol-mediated T cell activation or suppression. By looking at the signal transduction events of ethanol-treated T cells and comparing it to that seen in normal activation, we hope to understand the mechanism underlying ethanol-mediated immunosuppression.

In this chapter we investigate how T cells respond to an acute (5-15 minutes) exposure to ethanol and investigate the signal transduction events that are induced. For these studies we use the Jurkat-Raji cell system as a model system for studies of T cell activation. Jurkat cells are a human helper T lymphoma line that has been widely used to study T cell signaling and activation [109]. However, the antigen specificity of the Jurkat TcR is unknown. To circumvent this problem we used Raji cells (a cell line derived from B cells) that are coated with Staphylococcus Enterotoxin E (SEE)[110]. The SEE binds to both the MHC of Raji cells and the TcR of the Jurkat cells thus mimicking the ligation of TcR by MHC-peptide. This provides a positive control for comparison to mixtures of Raji and Jurkat cells in the absence of SEE where there should be no activation. Treatment of Jurkat-Raji mixtures with ethanol but no SEE can then be used to test for the activating effects of ethanol.

Using the Jurkat – Raji system we present data showing that that ethanol exposure activates Jurkat cells and induces signaling events that broadly parallel those seen in SEE-stimulated cells. Evidence for ethanol induced activation is seen in the increased

adhesion between Jurkat and Raji cells in the presence of ethanol. This adhesion is lost in the presence of the Src family kinase inhibitor PP2, or if mutant cell lines lacking the integrin LFA-1 (Jβ-2.7[111]), or the adapter protein LAT (ANJ-3[112]) are used. Furthermore, using markers for the immune synapse, we also show that ethanol causes formation of an immune synapse between Jurkat and Raji cells in the absence of SEE. Formation of this synapse correlates with tyrosine phosphorylation of the adapter protein LAT. Finally using the human cytotoxic T cell line TALL-104[113] we show that ethanol stimulation leads to release of granzymes as measured by assaying the release of BLT esterase.

MATERIALS AND METHODS

Reagents and Media:

Roswell Park Memorial Institute (RPMI) medium 1640, Iscove's Modified Dulbecco's Medium (IMDM), penicillin-streptomycin, glutamine and sodium pyruvate were purchased from Invitrogen (Carlsbad, CA). Heat inactivated Fetal Bovine Serum (HI-FBS) was obtained from Atlas Biologicals (Fort Collins, CO). Recombinant Human IL-2 was obtained from Chemicon International (Temecula, CA). Partially purified Staphylococcus Enterotoxin E (SEE) was purchased from Toxin Technology (Sarasota, FL). Calcium dyes indo-1 AM and fura-2 AM, the CRAC channel inhibitor, 2-Aminoethyl diphenyl borate (2-APB), poly-L-lysine (58 kDa MW), Tween-20, Triton X-100, Sodium Dodecyl Sulfate (SDS), Ethylene diamine tetracetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), and Tris were purchased from Sigma (St. Louis, MO). indo-PE3 AM and fura-PE3 AM leakage resistant isoforms of indo-1 and fura-2 were obtained from Teflabs (Austin, TX). Criterion nitrocellulose/filter paper sandwiches for immunoblotting were purchased from Bio-rad (Hercules, CA). Kodak BioMax light film

was purchased from Kodak USA (Rochester, NY). Super Signal Pico Chemilumiescent reagent was purchased from Pierce biotechnology (Rockford, IL). Paraformaldehyde was purchased from Aldrich (Milwaukee, WI). The nuclear and DNA counter-stain 4',6-diamidino-2-phenylindole (DAPI), Cell Tracker Green, Cell Tracker Blue and ProLong® Gold Anti-fade mounting media were purchased from Molecular Probes (Eugene, OR). The Src family kinase inhibitor PP2, 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB or Ellman's reagent) and N-α-benzyloxycarbonyl-L-lysinethiobenzyl ester (BLT) were purchased from Calbiochem (San Diego, CA). All other reagents were purchased from Sigma (St. Louis, MO).

Antibodies:

Mouse monoclonal anti CD3, anti Vβ-8, anti NFAT-1, anti PLC-γ, anti PKC-θ and anti-ADAP mAbs were purchased from BD-Pharmingen (San Diego, CA). Mouse Anti phospho-LAT mAb was purchased from Cell Signaling Technology (Danvers, MA). Rabbit anti LAT was purchased from Upstate (Charlottesville, VA). Rat anti α-tubulin was purchased from Chemicon International (Temecula, CA), while mouse anti β-tubulin and Cy-3 conjugated rabbit anti γ-tubulin were from Sigma (St. Louis, MO). All fluorescently conjugated anti mouse and anti rabbit antibodies were purchased from Jackson Immuno laboratories (West Grove, PA).

Cell Lines:

Jurkat (clone E6-1) human peripheral blood leukemia helper T cell line, Raji human Burkitt's lymphoma B cell line, and TALL 104 human acute lymphoblastic leukemia cytotoxic T cell line were obtained from ATCC (Manassas, VA). LAT deficient Jurkat cells (ANJ3) were a generous gift from Dr. Lawrence E. Samelson (National Cancer Institute at the National Institutes of Health). LFA-1 deficient Jβ-2.7 Jurkat cells

were a generous gift from Dr. Timothy Springer (Department of Pathology, Harvard Medical School).

Cell Culture:

Jurkat and Raji cells were cultured in RPMI medium 1640 containing 10% HI-FBS, 50 μM β-mercapto-ethanol, 24 mM NaHCO3, 1 mM pyruvate, and 1 mM glutamine. TALL 104 cells were cultured in IMDM containing 20% FBS and 100 units/μL recombinant human IL-2. All cells were grown in a 37°C incubator with 5% CO2. Media was replenished every two to three days.

Preparation of Poly-L-lysine coated coverslips:

No. 1 Glass coverslips (20 X 20 cm) were immersed in a 10% (v/v) solution of KOH (from a saturated stock solution) in Ethanol for 1 hr. The coverslips were washed by repeated immersion in distilled/de-ionized water (dd-H_2O). Excess water was wicked off on a paper towel and a small amount (25μL) of a 50mg/mL solution of 56KDa poly L-Lysine was applied to the cover slip and gently spread using a clean glass Pasteur pipet. The coated cover slip was washed again; excess water wicked off with a paper towel, covered to prevent any dust from settling and air dried at room temperature. Poly-Lysine coated coverslips were always freshly prepared a day ahead of an experiment. The dried coverslips were then each placed in a well of a six well plate where the cells were plated, fixed and immuno-stained.

Labeling of the Jurkat cell golgi

To label the Jurkat cell golgi, Jurkat cells were incubated for 15 minutes with Alexa-Fluor 594-labeled cholera toxin subunit B at a concentration of 50 μg/mL. Following incubation, cells were washed 3 times in RPMI media with no serum.

Conjugation of Jurkat and Raji cells

For experiments involving ethanol mediated pairs, Raji cells were incubated with 1μM Cell Tracker Blue and incubated for 15 minutes at 37°C, 5% CO_2. The labeled cells were then washed twice, counted and resuspended at 2 X 10^7 cells/mL in RPMI containing 1% FBS. E6-1 Jurkat cells were also counted, washed and resuspended at 2 X 10^7 cells/mL in the same medium. A 50uL aliquot of Jurkats was mixed with an equivalent volume of Raji cells. Then, 100μL of a 2X ethanol stock solution in RPMI with 1% FBS was diluted into the cell suspension, mixed, plated on poly lysine coverslips and incubated for 15 minutes at 37°C, 5% CO_2 to allow conjugation. As a control 100uL of plain RPMI with 1% FBS was added to another suspension of Jurkat Raji cells, mixed and plated as described.

For TcR mediated Jurkat Raji pairing (positive control), an aliquot of Raji cells was incubated with 2μg/mL SEE for 30—45 minutes. These cells were also labeled with Cell Tracker Blue as described above. The Raji cells were then washed twice resuspended at 1 X 10^7 cells/mL in RPMI with 1% FBS, mixed with an equivalent amount of Jurkat cells, plated on coverslips and incubated for 15 minutes as described above.

Preparation of Fixative

1 gram of parafomaldehyde was dissolved by boiling in 10mL dd-H_2O in the presence of 50uL 2N NaOH solution to give a 10% w/v formaldehyde solution. The formaldehyde solution was then diluted to 3.7% in PBS containing 1mM Calcium and 5mM Glucose.

Immunostaining

Coverslips with adhered cells were then fixed in this solution for 30 minutes at room temperature. The cells were briefly washed thrice in PBS and permeablized with ice cold 1:1::Acetone:Methanol and incubated on ice for 15 minutes. The cells were then washed thrice for 5 minutes each on an orbital shaker at 75 RPM with PBS and blocked for 30 minutes with 5% goat serum, 0.1% Tween-20 in PBS. The cells were once again washed thrice for 5 minutes each on an orbital shaker then incubated with the primary antibodies for one hour. Subsequently, the cells were washed 6 times (5 minutes each on orbital shaker) in PBS, incubated with the secondary antibody, washed again as before [51], and mounted on glass slides using the Prolong® Gold mounting media[114].

Image Acquisition and Processing

Images were acquired using a Nikon Diaphot 200 Inverted microscope using a Hamamatsu Orca CCD camera (Hamamatsu Corp., Bridgewater, NJ). Z-axis stacks consisting of 256 successive images were acquired using a MAC 2000 Z-axis focus controller (Ludl Electronic Products, Hawthorne, NY) and a custom image acquisition plug-in written for ImageJ [51]. Point spread functions for fluorescein and rhodamine fluorophores were generated using 0.2 micron fluorescent micro spheres obtained from Molecular Probes (Eugene, OR) [114]. Images were de-convolved using the maximum likelihood algorithm of XCOSM adapted to run on a PC [115].

Cell stimulation and Immunoblotting

E6-1 Jurkats were counted washed and resuspended at 5 X 10^6 cells/mL. 1 mL aliquots of cells in 1.5 mL centrifuge tubes were either left unstimulated or treated with various concentrations of ethanol for 5 minutes at room temperature. For positive control, an aliquot of Jurkat cells was stimulated with 0.5 μg of anti Vβ-8 mAb, incubated for 1

minute, then treated with 0.5 μg of an un-conjugated goat anti mouse Ab and incubated for 1 more minute at room temperature.

Following incubation, the cells were pelletted in the cold (4^{o}C) in a VWR desktop micro-centrifuge. Following centrifugation, the supernatant was removed by aspiration as the cells were lysed immediately in 400uL of hot 2X sample buffer (20 mM Tris (pH8.0), 2 mM EDTA, 2 mM Na_3VO_4, 20 mM DTT, 2% SDS, and 20% glycerol) [116]. The samples were then homogenized by through a 21-ga syringe needle, heated in a boiling water bath for 5 minutes and cleared by centrifugation at 250 X g.

Proteins were resolved by SDS-PAGE, and then transferred to a 0.2μm nitrocellulose membrane. The blot was blocked in 5% non-fat powdered milk solution in Tween-20 containing Tris Buffered Saline (T-TBS) (25 mM Tris/137 mM NaCl/2.7 mM KCl/0.025% Tween-20, pH 7.4). The membrane was incubated with agitation for 1 hr with the appropriate primary antibody diluted in blocking solution and subsequently washed thrice in T-TBS for 10 minutes each with agitation. The membrane was then incubated with a Horse Radish Peroxidase (HRP) conjugated secondary antibody for 1 hour before being washed thrice in T-TBS for 10 minutes each with agitation. The blots were developed using the Pierce Super Signal Pico Chemilumiescent development kit and detected by exposure to Kodak Biomax HR film.

BLT Esterase Assay

TALL-104 cells were counted, washed and resuspended at 2 X 10^5 cells/mL in DMEM supplemented with 10mM HEPES and 5% HI-FBS. The cells were plated at 50μl/well in a 96 well flat bottomed micro-titer plate using an 8-channel pipetter. Cells were stimulated with various concentrations of ethanol ranging from 25 to 400mM by adding 50μl of media containing twice the final concentration of ethanol. Unstimulated cells were given 50μl of media, as were cells that would be used to determine the total

cellular content of the enzyme by solubilizing with 0.1% Triton-X100 just before adding the BLT solution. Cells destined for TcR stimulation were plated in wells pre-coated for 8 minutes with 0.5μg/ml anti Vβ-8 mAb in DMEM without serum. The final concentration of cells was 1 X 10^5 cells/ml.

The cells were then incubated for 4 hours at 37°C and 5% CO_2, gently resuspended and centrifuged for 5 minutes at 250 rpm [117]. 50ul aliquots of supernatant were then transferred to a new plate containing equivalent volumes of the BLT solution (0.4mM BLT, 0.4mM DTNB) in DMEM supplemented with 10mM HEPES and 5% HI-FBS (final concentration of BLT solution was 0.2mM BLT, 0.2 mM DTNB) [118]. The mixture was incubated for 20 minutes at 37°C and absorbance was measured at 412nm compared to blank solution (DMEM with 10mM HEPES and 5% HI-FBS) that was treated exactly as experimental .

RESULTS

One way to examine T cell activation is to use markers at the immune synapse. To determine if ethanol induced changes at the contact site typical of T cell activation we first treated a 1:1 mixture of Jurkat and uncoated Raji cells with ethanol to see if ethanol exposure would cause the Jurkat and Raji cells to pair. We found that ethanol induces pairing between Jurkat and Raji cells to a higher level than that seen in the absence of ethanol (Figure 2.1 A & B). Investigating further, we found that ethanol mediated adhesion increases with increasing concentration from 5mM onwards (Figure 2.2). This pairing peaks at around 25 mM ethanol before decreasing at higher concentrations.

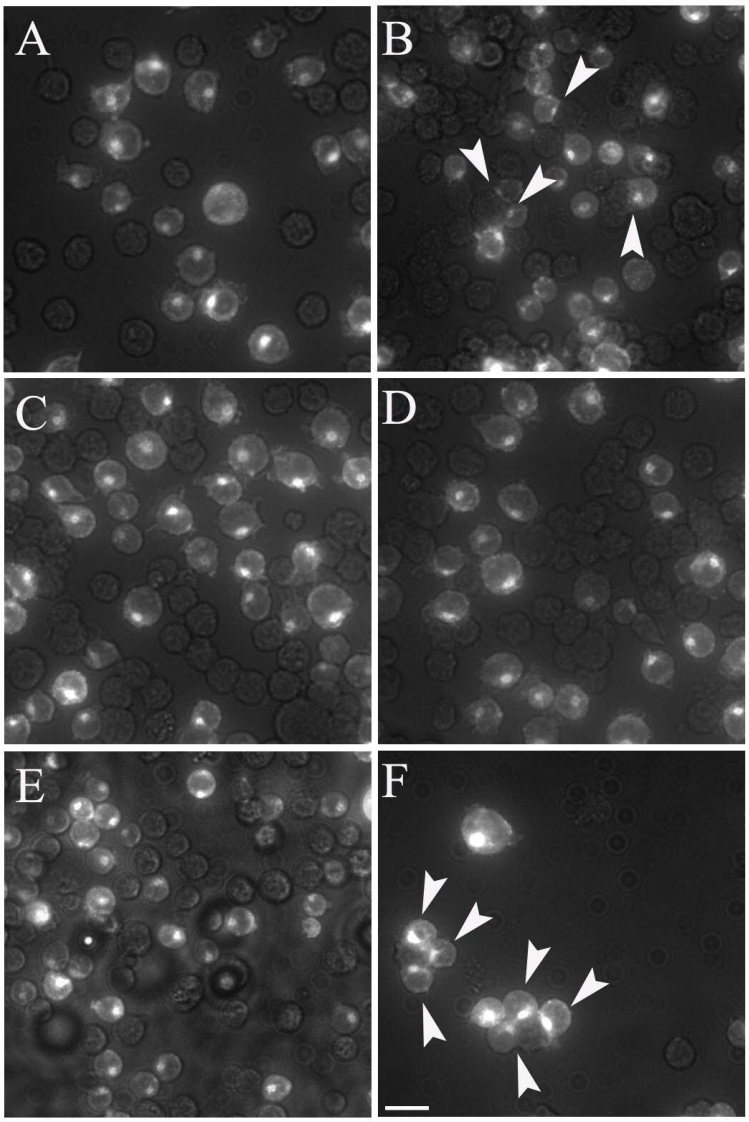

Figure 2.1: Pairing of Jurkat to Raji cells under various conditions.

Jurkat cells were loaded with Alexa 594-cholera toxin-B and mixed with unlabelled Raji cells (A—E). In panel **A** Jurkat cells were mixed with Raji cells in the absence of ethanol. In panel **B** Jurkat cells were mixed with uncoated Raji cells in the presence of 100 mM ethanol and paring can be seen (arrow heads). Ethanol-mediated pairing was not seen with Jβ-2.7 LFA-1-deficient Jurkat cells (C) or with, ANJ-3 LAT-deficient Jurkat cells **(D)**. Pairing was also not seen when Jurkat-Raji mixtures were treated with both ethanol and the Src family kinase inhibitor PP2 (E). Jurkat pairing with SEE-coated Raji cells (positive control) is shown in **(F)** for comparison. Bar = 10μm.

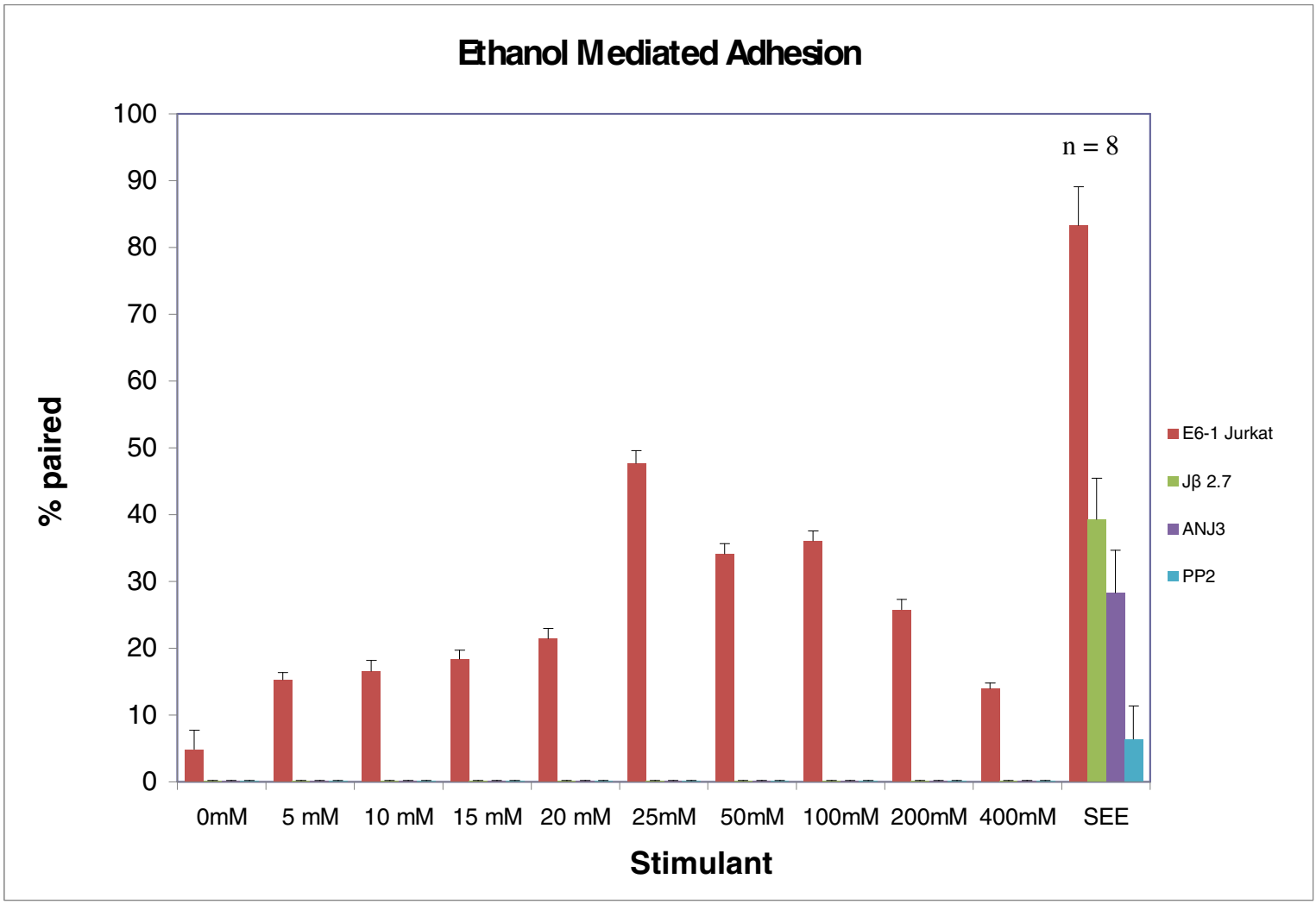

Figure 2.2: Ethanol induced pairing between Jurkat and Raji cells

Mixtures of Jurkat and uncoated Raji cells (1:1) were exposed to ethanol as indicated and the percentage of pairs were scored. Pairing between Jurkat and Raji cells was seen to increase with increasing concentrations of ethanol up to 25mM before decreasing at higher concentrations. Jurkats paired with SEE-coated Raji cells were used as a positive control. Little pairing was seen when Jurkat cells were treated with the Src family kinase inhibitor PP2, or when mutant LFA-1-deficient (Jβ-2.7) or LAT-deficient (ANJ-3) Jurkat cell lines were used. Error bars show standard error where n = number of replicates.

Having observed that ethanol induced adhesion we next sought to determine what adhesion molecule might be involved. One of the likely candidates is LFA-1 because it mediates initial cell-to-cell contact between the T cell and target APC allowing the TcR to come in contact with the MHC[45]. To test this possibility we compared LFA-$1^{-/-}$ Jurkat cell line Jβ-2.7 [111] to normal Jurkat cells to see if there was a difference in adhesion they would pair with Raji cells in ethanol. We observed no pairing between the LFA-$1^{-/-}$ Jurkat and Raji cells at any concentration of ethanol (Figure 2.1 C).

Since LFA-1 clustering normally is a consequence of inside-out signaling, we next asked if formation of adhesion complexes depended on signaling. This was tested by comparing adhesion using the LAT-deficient cell line ANJ-3 [112] to that seen with normal Jurkat cells. The results showed that LAT-deficient cells did not pair with Raji cells after exposure to ethanol (Figure 2.1 D). Since LAT normally is activated by a cascade of tyrosine phosphorylation triggered by activation of the Src kinase Lck[45], we compared adhesion in the presence and absence of the Src family kinase inhibitor PP2, [119]. The results showed that PP2 blocked the ethanol stimulated adhesion between Jurkat and Raji cells (Figure 2.1 E).

Having found some evidence for activation of Jurkat cells by ethanol we next looked at the distribution of LFA-1 in ethanol-mediated pairs compared to that seen in SEE-mediated pairs by immuno-staining. The results show that LFA-1 clustered at the synapse in ethanol-treated pairs (Figure 2.3 B, C) and organized into a ring (Fig 2.3 E, F) similar to that observed in Jurkat cells paired with SEE coated Raji cells (Figure 2.3 D & G).

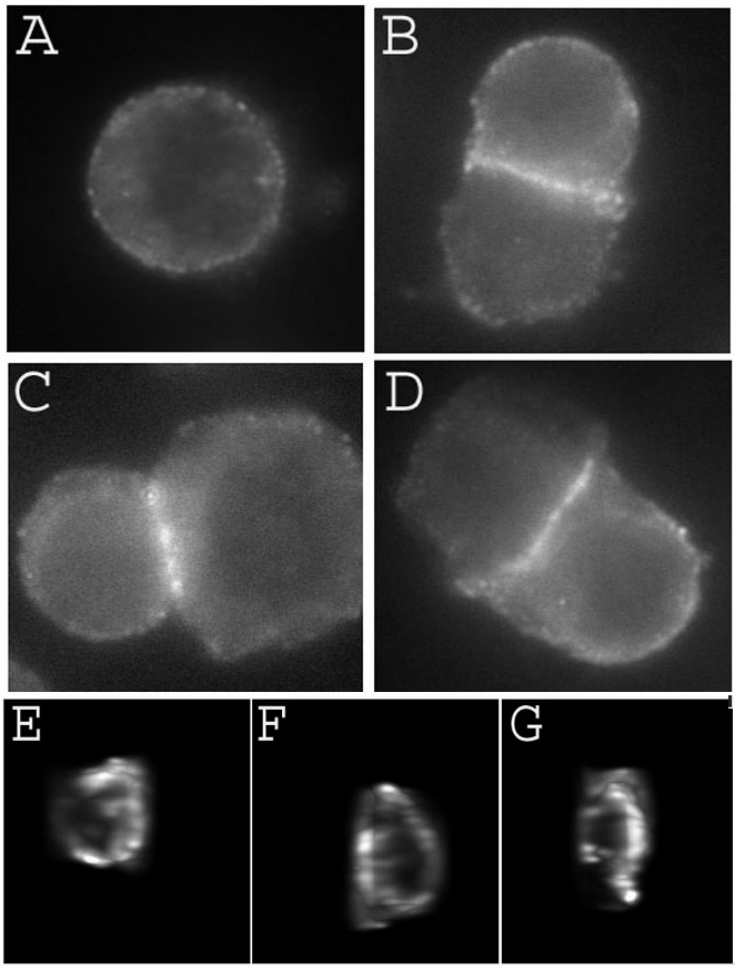

Figure2.3: Ethanol induces LFA-1 clustering and ring formation at the synapse.

Ethanol-treated Jurkat Raji cell pairs were fixed and stained for LFA-1. **(A)** In unstimulated cells LFA-1 is seen distributed uniformly over the surface. In pairs treated with either 25mM **(B)** or 100 mM ethanol **(C)**, we observed LFA-1 clustering at the site of contact between the Jurkat and Raji cells. This clustering of LFA-1 is similar to that seen in Jurkats paired with SEE-coated Raji cells **(D)**. Moreover, when image stacks are processed by computerized 3D reconstruction it becomes apparent that LFA-1 organizes into a ring at the contact site **(E & F)** similar to that seen with SEE-mediated pairs **(G)**.

F-actin is also critical to the activation of T cells as it is required for adhesion to target cells, signaling and the formation of the immunological synapse [120]. Actin

filaments also provide a scaffold for signaling complexes. This scaffold in turn may also play a role in recruiting or stabilizing specialized membrane domains enriched in glycol lipids and signaling molecules involved in T cell activation. [121]. The active form of LFA-1 binding to its receptor ICAM-1 results in LFA-1 mediated F-actin reorganization that in turn results in enhanced adhesion of T cells to targets [122].

We therefore wanted to determine whether F-actin also accumulates at the synapse of ethanol-treated Jurkat-Raji pairs. To do this, Jurkat-Raji mixtures were treated with various concentrations of ethanol and then fixed and stained with rhodamine-phalloidin. (Figure 2.4 A & B) The data show that F-actin clusters at the contact site in patches at lower concentrations of ethanol (25mM; Figure 2.4 D) and organizes into a ring at higher concentrations of ethanol (100mM; Figure 2.4 E) similar to that seen in the SEE induced synapse (Figure 2.4 F).

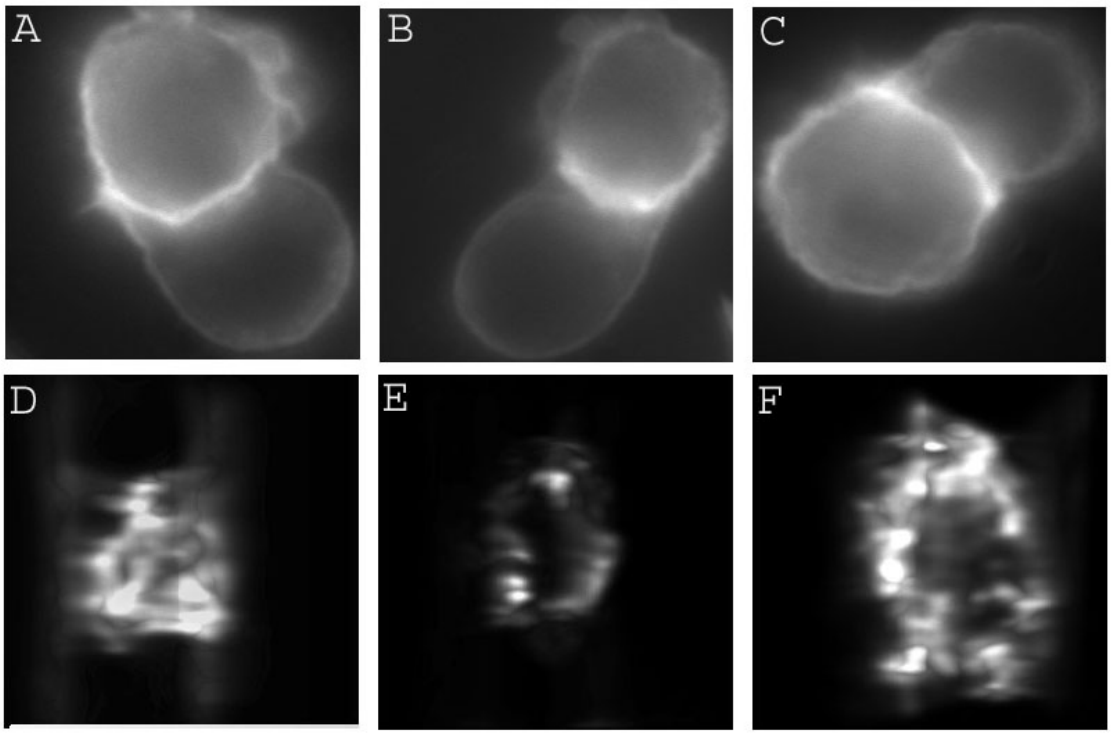

Figure 2.4: F-accumulates at the contact site and forms a ring both in ethanol and SEE mediated Jurkat-Raji pairs.

Jurkats were paired with Raji cells in the presence of either SEE or ethanol. These mixtures were then fixed and stained with rhodamine-phalloidin. The results show the presence of F-actin clustering in both **(A)** 25 and **(B)** 100 mM ethanol which was similar to the clustering seen when Jurkat-Raji pairs were stimulated by SEE (C). It is interesting to note that actin organizes into a ring when Jurkat-Raji mixtures are stimulated with 100mM ethanol **(E)** or SEE **(F)** but not when stimulated with 25mM ethanol **(D)** suggesting a dose dependent effect of ethanol.

The accumulation of LFA-1 at the synapse is not a passive event. It requires inside-out signaling mediated by several signaling components. One of these components is the scaffold protein ADAP [123] which is sometimes referred to as Fyb/SLAP-130. Previous work done in our lab has shown that in Jurkat/SEE-Raji pairs, ADAP translocates from the MTOC region to the synapse where it is organized into a ring[114]. Work in our lab has also shown that ADAP plays a vital role in the translocation of the

Microtubule Organizing Center (MTOC) to the synapse[114] which in turn controls the directed secretion of cytolytic granzymes by cytotoxic T lymphocytes to kill targets [51]. We have found that at the synapse of ethanol-treated Jurkat-Raji pairs, ADAP clusters (Figure 2.5 A & B) in a ring-like pattern (Figure 2.5 D & E) similar to that seen in SEE-Raji mediated pairs (Figure 2.5 C & F).

The immune synapse is organized into two distinct regions. The outer ring-like region is known as the pSMAC while the central region is referred to as the cSMAC. So far we have only studied the markers for synapse formation that organized into ring-like patterns and segregated into the pSMAC. While this was a strong indicator that ethanol stimulation resulted in nearly normal synapses, we still had to determine whether the overall architecture of the immune synapse was similar both in SEE and ethanol-mediated synapses. Proteins like ADAP and LFA-1 are associated with the pSMAC and organize themselves into ring-like patterns on the contact site. Other proteins such as CD-81 and CD-82, which are members of the tetraspanin family of cell surface proteins, cluster into the cSMAC upon T cell activation. Tetraspanin proteins are small cell surface proteins that consist of 4 trans-membrane domains[124]. CD-82 has been shown to have a co-stimulatory role in T cell activation[125] possibly by interacting with raft domains and the actin cytoskeleton in order to help regulate the organization of the immune synapse [126]. CD-82 has also been reported to enhance LFA-1/ICAM-1 interactions thus promoting T cell APC adhesion [127].

We therefore studied whether ethanol stimulation would result in CD-82 clustering in a pattern similar to that seen in SEE mediated pairs. We observed that the pattern of CD-82 clustering in ethanol-treated Jurkat Raji pairs (Figure 2.6 B & D) was the same as that in SEE-stimulated pairs (Figure 2.6 C).

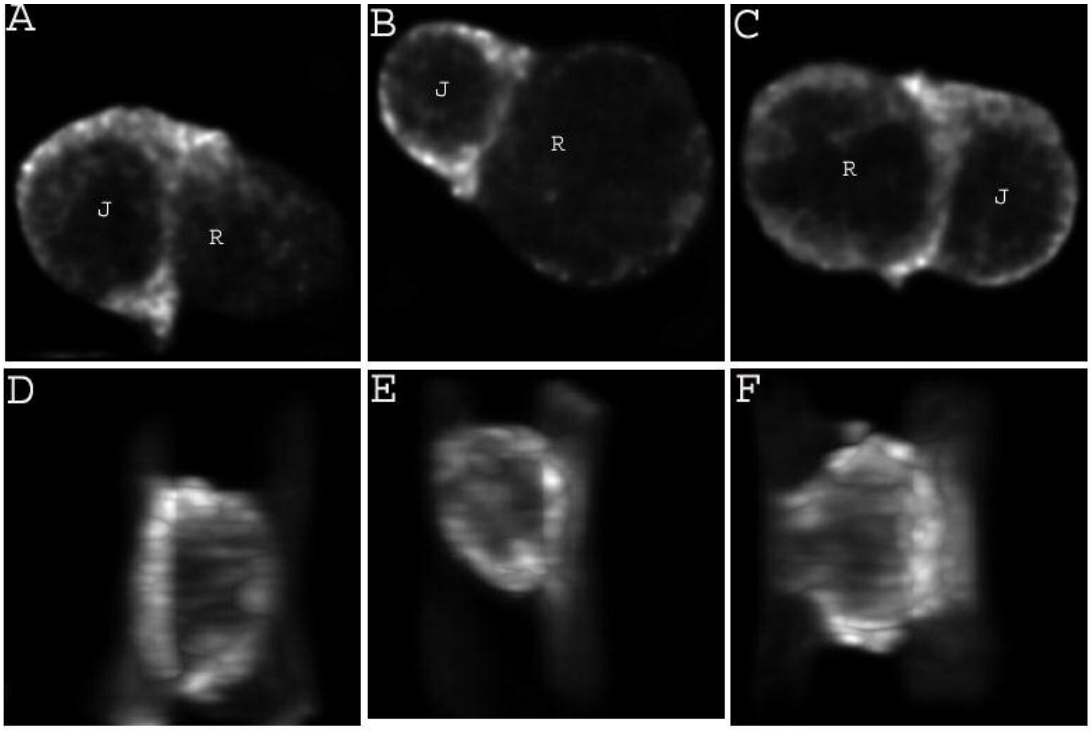

Figure 2.5: ADAP clusters at the synapse and forms a ring both in ethanol and SEE stimulation.

Treatment of Jurkat-Raji mixtures with either 25mM **(A&D)** or 100mM **(B&E)** ethanol causes ADAP to cluster at the periphery and form a ring at the synapse similar to that seen in Jurkat-Raji pairs stimulated with SEE **(C&F)**.

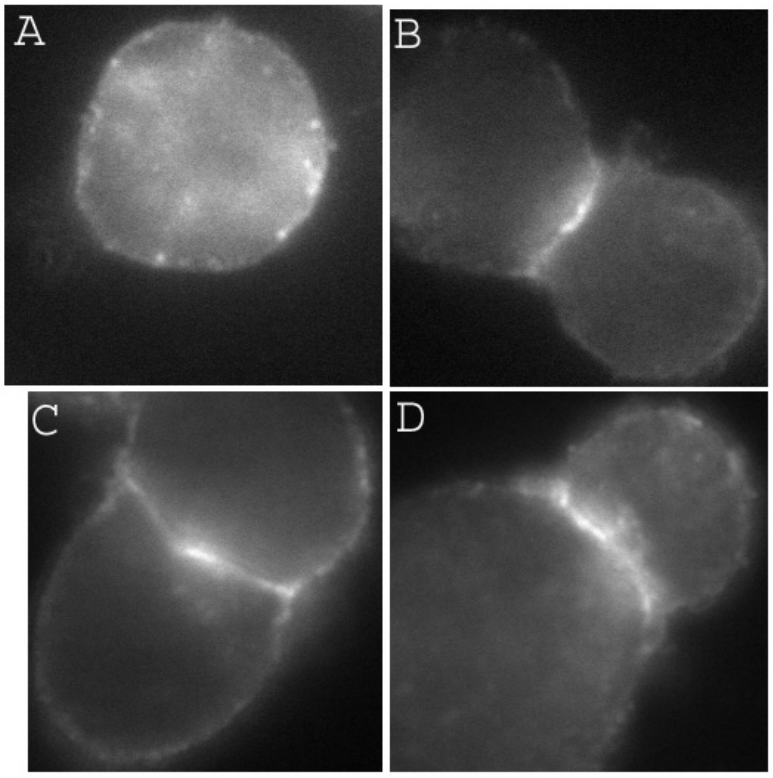

Figure 2.6: CD-82 clusters normally in ethanol mediated pairs.

Unpaired Jurkat cells show a uniform distribution of CD-82 **(A).** Jurkat-Raji pairs whether stimulated with 25mM **(B)**, 100mM **(D),** or SEE(**C)** show similar pattern of CD-82 accumulation at the synapse.

The normal clustering of proteins tested so far in the ethanol mediated synapse made it clear that signaling cascades were being induced in Jurkat cells exposed to ethanol. We had noted that the Src family tyrosine kinase inhibitor PP2, a well-known inhibitor of T cell activation completely eliminates pairing between Jurkat and Raji cells in ethanol (Figure 2.1E). Therefore we decided to look at the level of tyrosine phosphorylation in cells stimulated with ethanol as a measure of activation.

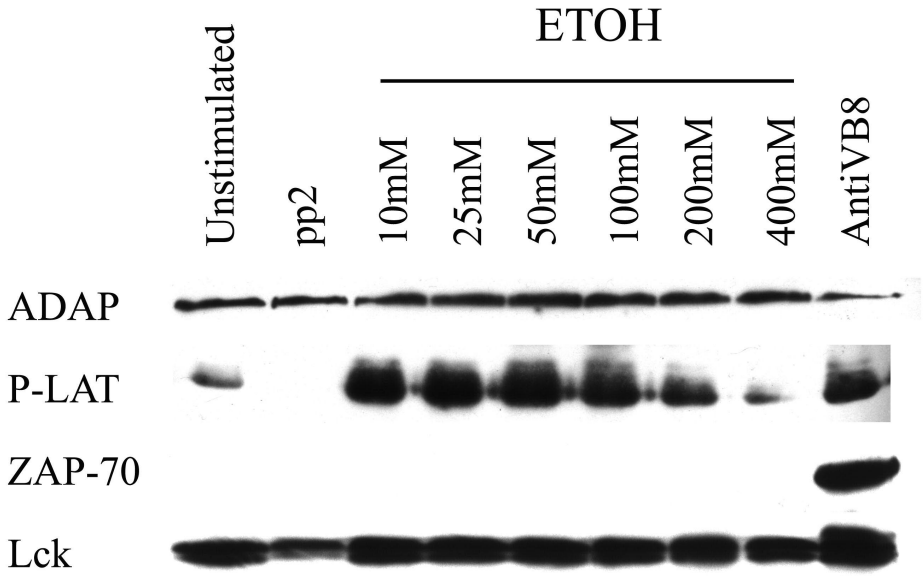

Figure 2.7: Ethanol causes tyrosine phosphorylation of LAT.

Jurkat cells were treated with various concentrations of ethanol (10 to 400mM) for 5 minutes. The cells were then lysed in hot SDS sample buffer, separated by SDS gel electrophoresis and analyzed on blots using a phospho-LAT specific mAb. The cells treated with ethanol show increased LAT tyrosine phosphorylation. However, no increase in phosphorylation is detected for either ZAP-70 or Lck. For comparison, 10μM **PP2** was used as a negative control to block activation of Lck and **Anti Vβ-8** was used to cross-link the TcR (positive control). The blot was also probed with ADAP for a loading control.

The Linker for Activation of T cells (LAT) is a type-III trans-membrane protein with a long cytoplasmic tail that contains 9 tyrosine based motifs [45]. When phosphorylated by ZAP-70 these motifs serve as docking sites for specific SH2 proteins including PLC-γ, PI-3 kinase, Grb2 and Gads. As such, LAT functions as a bridge between proximal and distal portions of TCR signaling [48] and plays a role in mediating $Ca2^{+}$ flux, activation of Ras/Erk and NFAT/AP-1 pathways as well as PI-3 kinase activation [128-130]. LAT contains a palmitoyl tail, which allows it to concentrate in the lipid rafts where it interacts with and keeps Lck in a closed inactive conformation [131].

We have already established a role for LAT in ethanol mediated T cell activation with the observation that LAT-deficient ANJ-3 cells do not adhere with Raji cells upon exposure to ethanol. Moreover LAT shows the strongest band in phosphotyrosine westerns of activated T cells thus making its detection and the measurement of small changes (as would be expected with ethanol stimulation) in phosphorylation easier [48].

To look at the effects of ethanol on LAT, we incubated Jurkat cells with various doses of ethanol, lysed with hot 2 X sample buffer, resolved by SDS-PAGE and analyzed phosphorylation by immunoblotting with an anti-phosphoLAT antibody. We see that ethanol concentrations in the range of 10—100 mM clearly induced LAT phosphorylation with reduced phosphorylation at higher concentrations (Figure. 2.7). In normal activation of T cells, LAT is usually phosphorylated by ZAP-70 which in turn is phosphorylated and activated by the Src family kinase Lck. If LAT was being phosphorylated by the normal T cell activation cascade, we would expect to see an increase in phosphorylation of both proteins since this normally occurs in conjunction with their activation. However, the data show that antibody stimulation caused an increase in the tyrosine phosphorylation of ZAP-70 but ethanol treatment, surprisingly, did not. Moreover tyrosine phosphorylation of Lck also increased only upon stimulation

with antibody and not upon stimulation with ethanol. This suggests that ethanol–stimulated phosphorylation of LAT might be caused by other Src family kinases like Fyn.

This tyrosine phosphorylation of LAT was also confirmed by immunostaining ethanol-stimulated Jurkat-Raji pairs with the same phospho-LAT antibody (Figure 2.8). This would help us to confirm that phosphorylated LAT clustered at the synapse in a pattern similar to that seen in SEE–stimulated pairs. The results showed that both in SEE and ethanol-treated pairs; phosphorylated LAT tends to accumulate at the contact site. Staining with β-tubulin allowed us to look at MTOC polarization in ethanol-mediated Jurkat-Raji pairs as another measure of T cell activation (Figure 2.9A). It was observed that ethanol-stimulated pairs showed MTOC polarization that increased in a concentration-dependant manner (Figure 2.9 B).

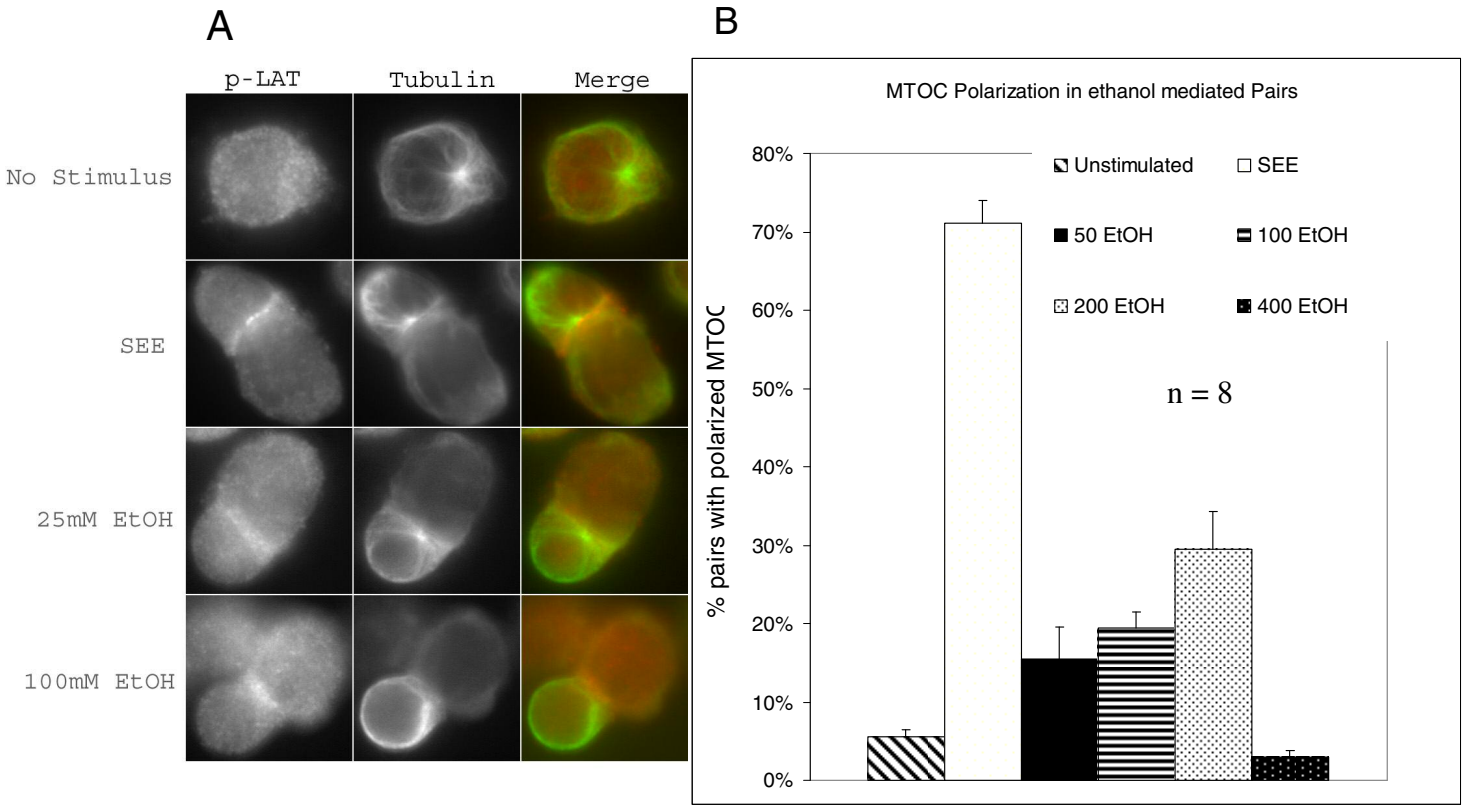

Figure 2.8: p-LAT immuno-staining of Jurkat-Raji pairs stimulated by SEE or Ethanol.

(A) Jurkat Raji pairs were treated with ethanol or SEE and then fixed and immunostained for phospho-LAT and β-tubulin and scored for MTOC translocation. Notice the tight phospho-LAT band formed at the synapse in a Jurkat SEE Raji pair. The clustering of phospho-LAT in ethanol- treated pairs is neither as bright nor so compact suggesting a weaker or incomplete activation induced by ethanol. The graph **(B)** shows the percentage of MTOC polarization in Jurkat Raji pairs. Error bars show standard error with n representing replicates.

Up till now we have done experiments only with Jurkat cells, a highly transformed human helper T cell line. While we have confirmed that ethanol activates early signaling events, we have as yet not determined if these signaling events lead to a productive response. The BLT esterase assay [118] provides a convenient way to determine whether cytotoxic T cells are activated by using cytolytic granzyme release as a marker for activation.

TALL-104 cells were incubated in concentrations of ethanol ranging from 25—50mM as well as low concentrations of antibody in 96 well plates for 4 hours. The cells were then centrifuged and aliquots of supernatant transferred to a fresh plate where they were incubated with the BLT media (0.2 M BLT, 0.2 M DTNB) for 30 minutes before reading the color change in DTNB using a plate reader[117]. Ethanol treatment resulted in the release of cytolytic granzymes to the same extent as treatment of cells with low doses (4—16ng/ml) of anti-TcR antibody (Figure 2.9). This is important because it shows that ethanol stimulation elicits the same outcome from cells as low doses of anti-TcR antibody. We shall see in chapter 3, these low doses of antibody also yield the same degree of calcium flux as was observed with ethanol stimulation.

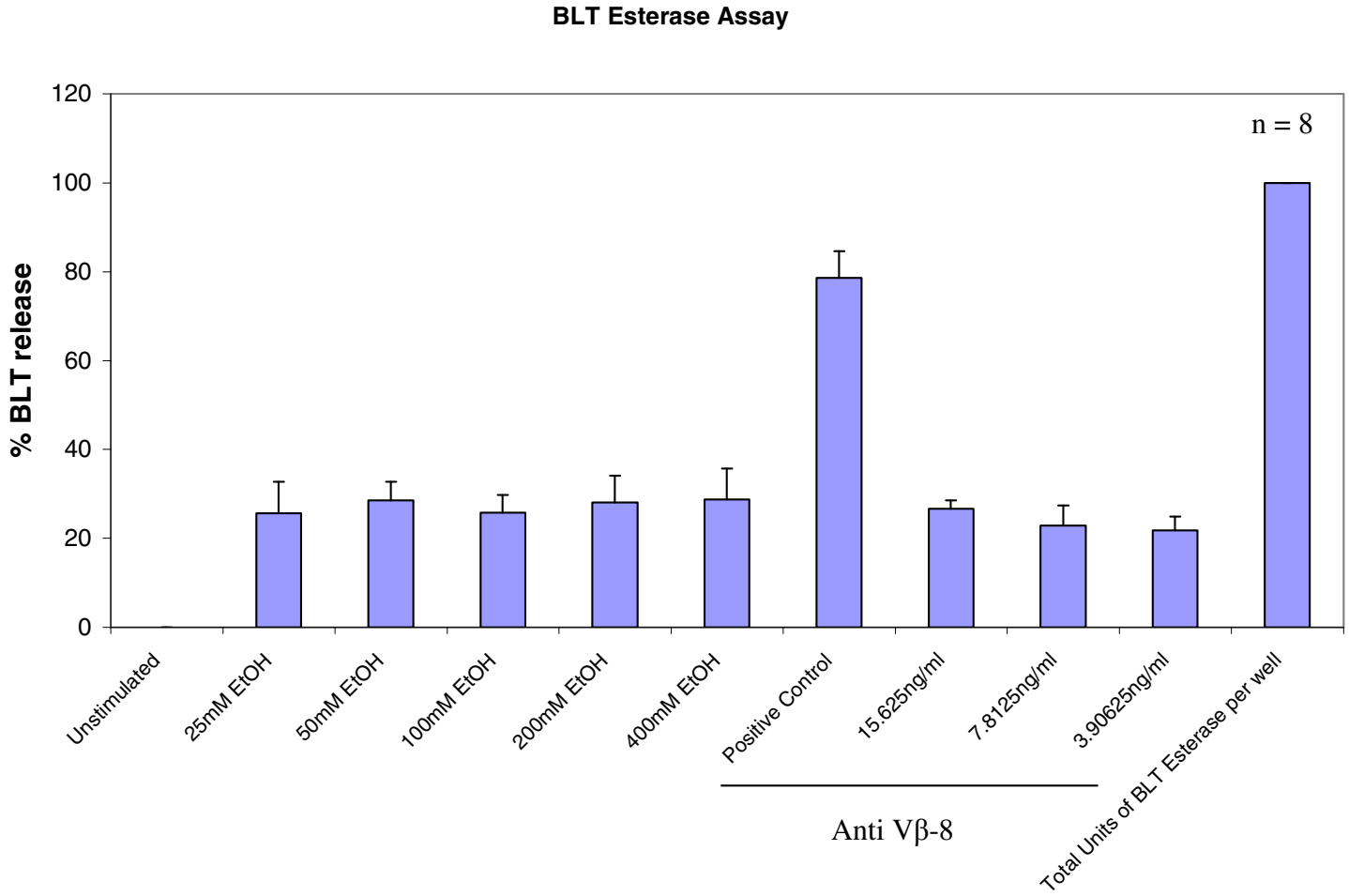

Figure 2.9: Ethanol stimulates cytotoxic T cells to release cytolytic granzymes.

TALL 104 cells were incubated without stimulation or in various doses of ethanol or antibody for 4 hours at 37°C in 96 well plates. The cells were then centrifuged and aliquots of supernatant were taken and assayed for cytolytic granzyme release. Various concentrations of ethanol cause the release of granzymes. The amount of granzyme activity is parallel to that seen in cells treated with low doses of anti TcR antibody. Error bars show standard error where n = replicates.

DISCUSSION

These studies demonstrate that ethanol exposure causes Jurkat T lymphocytes to adhere to uncoated Raji cells (Figure 2.1B). The adhesion of a T cell to an antigen-

presenting cells is a signal-mediated event that requires a reorganization of the cellular cytoskeleton[132]. Interactions between integrins like LFA-1 and ICAM-1 play a vital role in initiating contact between a T cell and an antigen-presenting cell [133]. Inside-out signaling in turn up-regulates these interactions. The failure of LFA-1-deficient Jβ-2.7 Jurkat cells to adhere to Raji cells when stimulated with ethanol suggested that ethanol induced LFA-1-mediated adhesions. This provides an interesting clue to the action of ethanol because LFA-1 clustering and adhesions normally depend on signaling events in the T cell and are associated with the formation of an immunological synapse.

By immunostaining ethanol-stimulated Jurkat-Raji pairs we saw LFA-1, ADAP and actin clustering at the contact site and organizing into a ring while CD-82 clustered at the center of the contact surface. We also observed that actin clustered into a patch at the contact site when Jurkat-Raji pairs were stimulated with 25mM ethanol rather than in the shape of a ring as seen with pairs induced by 100mM ethanol and SEE. This is the first and so far the only indicator of an incomplete or abnormal clustering of a marker of synapse formation that we have observed. This result is especially exciting because of reports that the adaptor ADAP regulates TcR-induced integrin clustering and activation[134] that in turn plays a role in actin polymerization [135]. This result suggests that at lower concentrations of ethanol, we may observe the incomplete clustering or lack of clustering of other markers of synapse-formation. If true, ethanol could prove to be an effective tool in dissecting the activation pathway and thus help in pinpointing the order in which the signaling complexes involved in T-cell activation are formed.

We also observed that a significant number of ethanol-induced pairs have polarized MTOCs in the Jurkat cells. In other words ethanol also induces the formation of the immunological synapse. This implied that ethanol was activating signaling cascades within the T cells.

The idea that ethanol stimulation leads to the activation of signaling cascades in T cells was further strengthened with the finding that ethanol does not induce pairing of LAT-deficient ANJ-3 Jurkat cells with Raji cells. Moreover, pre-treating Jurkat cells with the Src family kinase inhibitor PP2 also inhibits ethanol-stimulated pairing. By immunoblotting lysates from ethanol-stimulated Jurkat cells, we saw that ethanol leads to the tyrosine phosphorylation of LAT and that this phosphorylation is blocked by PP2. Finally, we have also shown that ethanol causes the release of cytolytic granules in cytotoxic T lymphocytes (TALL-104) using the BLT esterase assay.

The data discussed so far suggests that treatment with ethanol induces the same signaling events as TcR stimulation. These signaling events progress largely through tyrosine phosphorylation of catalytic and non-catalytic (adapter) proteins. One surprising aspect here was the lack of tyrosine phosphorylation seen in ZAP-70 and Lck but increased tyrosine phosphorylation of LAT as a result of ethanol stimulation. However pretreatment of Jurkat cells with the Src family kinase inhibitor PP2, results in the abrogation of ethanol mediated tyrosine phosphorylation of LAT. These data suggest that ethanol induces a different pathway to LAT phosphorylation than one seen in normal activation of T cells. However this altered pathway is likely still dependant on a Src family kinase.

It is interesting to note that in assays for pairing and MTOC polarization, we see that the respective responses increase with increasing concentrations of ethanol. However in tyrosine phosphorylation of LAT and the BLT esterase assay for cytolytic granzyme release, we do not observe an increase in the response with increasing concentrations of ethanol. Moreover the tyrosine phosphorylation of LAT as well as the release of cytolytic granzymes in response to ethanol is lower in magnitude as compared to controls stimulated with anti-TcR antibody. More experiments need to be done at lower

concentrations of ethanol to see at what concentration the phosphorylation of LAT and release of cytolytic granzymes is first seen and whether it increases with increasing concentrations of ethanol. The BLT esterase assay was done using the human cytotoxic T cell line TALL-104. Experiments assaying the tyrosine phosphorylation of LAT as well as the formation of the immune synapse in response to ethanol stimulation should be done to ascertain if the response of these cells is similar to that seen with Jurkat cells.

While we observe that ethanol-stimulated T cells show similar responses as normally activated T cells, we have yet to determine the basis of ethanol mediated action. In this regard the study of calcium flux within the cells provides an invaluable tool to see whether the activation events in both cases are similar in their origins. We can also investigate then whether ethanol exposure exerts any effects on subsequent TcR stimulation. This in turn would set the stage for a possible mechanism, for inducing immunosuppression in ethanol stimulated cells.

Chapter 3: Acute Ethanol and Calcium flux

INTRODUCTION

Studies reporting both activating and inhibiting effects of ethanol on T cells point to a possible important role for calcium flux in these cells. Based on the pathway and accompanying signals, $[Ca^{2+}]_i$ elevation can lead to T cell activation and proliferation or cause the onset of inactivation and anergy. Activation of T cells by antigen commences with an early wave of tyrosine phosphorylation, which leads to the activation of downstream signaling pathways including an increase in intracellular free calcium ($[Ca^{2+}]_i$). Calcium acts as an important trigger for transcription in the nucleus. The opening of CRAC channels lead to oscillations in $[Ca^{2+}]_i$ and the nature of these oscillations (slow vs. fast) determines which transcription factors are activated and which genes are transcribed [54]. It is important to note here that TcR stimulation is not the only way to induce $[Ca^{2+}]_i$ elevation [136]. For our studies we have used the Jurkat helper T lymphocytes, a lymphoma cell line that is widely used in T cell studies [109], and are especially useful because of the wide variety of mutant cell lines available. Furthermore, they have been extensively studied with regard to calcium signaling.

Here, we focus on calcium signaling as one of the important effects of ethanol. We observe concentration-dependent calcium transients due to ethanol that are comparable to those triggered by low doses of anti-TcR antibody. This is important because it allows us to compare ethanol-dependent signaling to that normally triggered through stimulating the T cell receptor. Analysis of the calcium signaling pathway indicates that ethanol-stimulated calcium transients depend on calcium entry and are likely due to opening of CRAC type calcium channels. The observed calcium transients set the stage in explaining the role of ethanol both in acutely activating T cells and

immuno-suppression in the long term. However, it seems clear that ethanol does more than simply raise calcium as evidenced by data showing that ethanol activates some of the same signaling events as seen in TcR-mediated activation.

MATERIALS AND METHODS

Reagents and Media:

Roswell Park Memorial Institute (RPMI) medium 1640, Iscove's Modified Dulbecco's Medium (IMDM), penicillin-streptomycin, glutamine and sodium pyruvate were purchased from Invitrogen (Carlsbad, CA). Heat inactivated Fetal Bovine Serum (HI-FBS) was obtained from Atlas Biologicals (Fort Collins, CO). Recombinant Human IL-2 was obtained from Chemicon International (Temecula, CA). Calcium dyes INDO-1 AM and FURA-2 AM, the CRAC channel inhibitor, 2-Aminoethyl-diphenyl-borate (2-APB), poly-L-lysine (58 kDa MW), Tween-20, Triton X-100, Sodium Dodecyl Sulfate (SDS), Ethylene diamine tetracetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), and Tris were purchased from Sigma (St. Louis, MO). Indo-PE3 AM and fura-PE3 AM leakage resistant isoforms of indo-1 and fura-2 were obtained from Teflabs (Austin, TX). The Src family kinase inhibitor PP2 was purchased from Calbiochem (San Diego, CA). All other reagents were purchased from Sigma (St. Louis, MO).

Antibodies:

Mouse monoclonal anti CD3 and anti Vβ-8 (catalog number 555604) [137], were purchased from BD-Pharmingen (San Diego, CA). Unconjugated Goat anti mouse secondary antibody was purchased from Upstate (Charlottesville, VA).

Cell Lines:

Jurkat (clone E6-1) human peripheral blood leukemia helper T cell line, Raji human Burkitt's lymphoma B cell line, and TALL 104 human acute lymphoblastic

leukemia cytotoxic T cell line were obtained from ATCC (Manassas, VA). LAT deficient Jurkat cells (ANJ3) were a generous gift from Dr. Lawrence E. Samelson (National Cancer Institute at the National Institutes of Health).

Cell Culture:

ANJ3 LAT deficient Jurkat, E6-1 normal Jurkat, and Raji cells were cultured in RPMI medium 1640 containing 10% HI-FBS, 50 μM β-mercaptoethanol, 2 g/l NaHCO3, 1 mM pyruvate, and 1 mM glutamine 100 units/ml penicillin, 100μg/ml streptomycin in a humidified 37°C incubator with 5% CO2.

Preparation of Poly-L-lysine coated coverslips:

No. 1 Glass coverslips (20 X 20 cm) were immersed in a 10% (v/v) solution of KOH (from a saturated stock solution) in Ethanol for 1 hr. The coverslips were washed by repeated immersion in distilled/de-ionized water (dd-H_2O). Excess water was wicked off on a paper towel and a small amount (25μL) of a 50mg/mL solution of 56KDa poly L-Lysine was applied to the cover slip and gently spread using a clean glass Pasteur pipette. The coated cover slip was washed again; excess water wicked off with a paper towel, covered to prevent any dust from settling and air dried at room temperature. Poly-Lysine coated coverslips were always freshly prepared a day ahead of an experiment.

Calcium Measurements

Fluorimeter measurements

E6-1 Jurkats were counted, washed and resuspended at a concentration of 1 X 10^6 cells/ml in pre-warmed HBSS with 25mM HEPES buffer, 5mM Glucose, 1% FBS, pH 7.2, and loaded with 1 μM indo-PE3 for 1.5—2 hours at 37°C. The cells were then washed once with HBSS, resuspended at a concentration of 1 X 10^6 cells/ml and incubated at 37°C for 10 minutes to allow complete de-esterification of the dye inside the

cells. Measurements were carried out in a PTI dual emission fluorimeter in a standard fluorimeter cuvette using 3ml aliquots of cells.

The dye was excited at 360nm with emitted fluorescent light monitored by two photomultiplier tubes at 404 and 485nm. The ratio of the 404 and 485nm signals provides a measure of the $[Ca^{2+}]_i$ [138]. The cells were kept in constant agitation using a magnetic stirrer. The resting $[Ca^{2+}]_i$ of cells was recorded, the cells were then stirred by pipetting up and down using a transfer pipette and $[Ca^{2+}]_i$ recorded again. The stimulant (ethanol or antibody) were then added, the cells and stirred and $[Ca^{2+}]_i$ was recorded for 400 seconds. To calibrate the signals we obtained the maximum (R_{max}) and minimum (R_{min}) values for the calcium ratio. To obtain R_{max}, 2μM of the calcium ionophore ionomycin was added to the cells. The cells were then lysed with digitonin to ensure saturation of the dye with Ca^{2+} present in HBSS. In order to obtain R_{min}, 10mM EGTA and 20mM Tris were added to the lysed cells. These calibration steps were done at the end of each run.

Imaging measurements:

The cells were counted, washed and resuspended at a concentration of 1 X 10^6 cells/ml in pre-warmed HBSS with 25mM HEPES buffer, 5mM Glucose, 1% FBS, pH 6.8 and loaded with 2μM FURA-PE3 AM for 1 hr at 37°C. For loading with FURA-2 AM, the procedure was the same except that the pH was 7.2 and the cells were incubated with the dye for 30 minutes[139]. Cells were then pelleted and resuspended in HBSS.

The cells were then added to a 22 mM square poly-L-lysine coated cover slip attached using silicon vacuum grease to the bottom of a 35mm diameter Petri dish containing a hole about 15mm in diameter and allowed to adhere. The coverslip and its mount were then placed in an aluminum water jacketed holder. The holder was mounted on the stage of a Zeiss IM-35 microscope. The temperature was held constant at 37°C by

circulating heated water through the cover slip holder and through coiled tubes circling the Nikon UV-F 40X/1.3NA objective lens.

Fluorescence ratio images were collected and processed essentially by the same process as described by Poenie and Tsien[140]. A PTI Deltascan dual monochromator light source was used to generate dual excitation wavelengths. Images were processed using the PTI Image Master system. Images were calibrated by dividing each raw ratio image by the ratio image obtained at the beginning of each experiment from a thin film of solution containing 25 μM fura-2 penta-potassium salt in 10 mM EGTA, 50mM HEPES, pH7.2. The resulting ratio images were expressed as a multiple of Rmin. For calculations of [Ca2+]i, Rmax was pre determined as described[141] and the equation (R-Rmin)/(Rmax-R) x K was used with K being a predetermined constant for the dye. Calcium levels are coded as pseudo-colored hues ranging from blue, which represents low calcium values to purple, which represents the high calcium levels. [Ca2+]i values were extracted from pseudo color images using NIH Image J software with PTI acquire image and multi-measure plug-in.[142].

RESULTS

To determine the effect of ethanol on the calcium flux within cells, initial measurements were carried out using Jurkat cell suspensions loaded with indo-PE3, a leakage resistant variant of the indo-1 family of fluorescent dyes [138, 139]. Upon binding calcium, indo-1 shifts its emission wavelength. Measuring emission at two wavelengths for indo-1 (bound and unbound dye) enables us to differentiate between real calcium fluxes, where the intensity of one wavelength would increase and the other decrease correspondingly, from artifacts where both wavelength intensities tend to increase or decrease together (Figure 3.1 A)[140, 143]. This is especially useful in measuring ethanol stimulated signals which are weaker than those obtained from TcR cross-linking. Also, taking the ratio of the two emissions give us a value proportional to the calcium level within the cell (Figure 3.1 B).

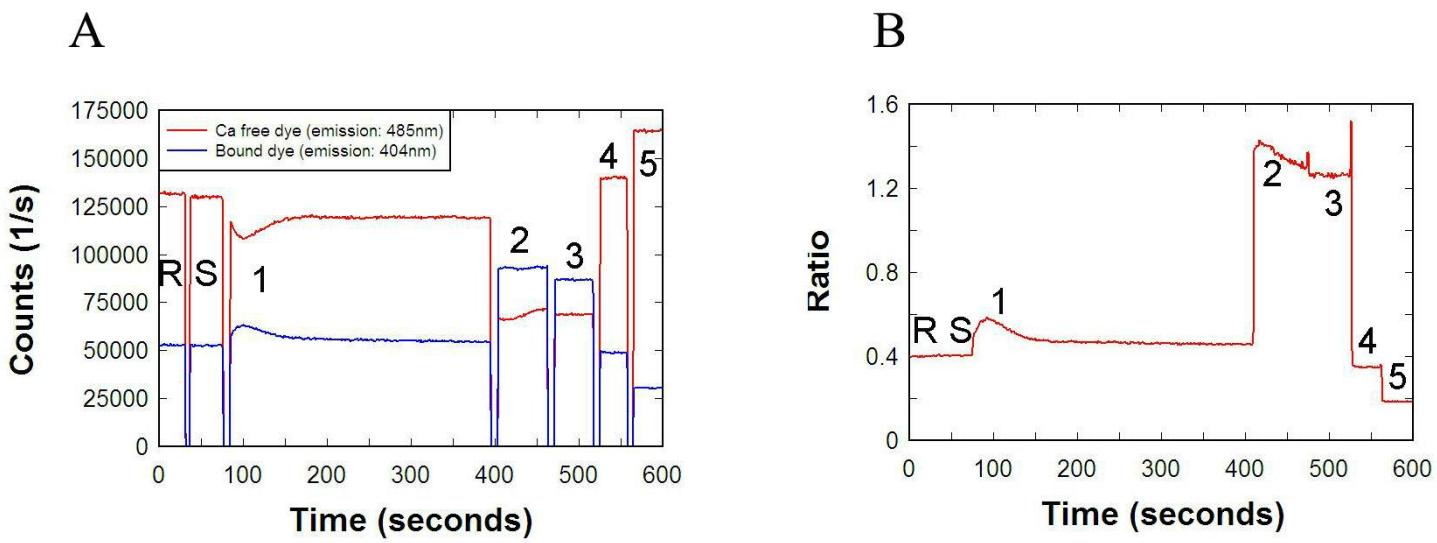

Figure 3.1: Dual Emission trace from indo-PE3 loaded Jurkat cells.

Jurkat cells were loaded with indo-PE3/AM for 1.5—2 hrs, before being washed once and incubated for 10 minutes to allow complete hydrolysis of the AM ester. The cells were then transferred to a fluorimeter cuvette and fluorescence emission was recorded at two wavelengths **(A)**. The essential parts of the experiment are marked. R: is the initial resting state of the cells. S is the level after the cells have been resuspended using a transfer pipette. The point labeled "1" shows the typical change in intensities of fluorescence at both wavelengths of the dye following the addition of 200mM ethanol. This reciprocal change in the fluorescence intensities represents a real change (in this case an elevation) in $[Ca^{2+}]_i$. Following the stimulation, the signals were calibrated by adding 2 μM of the calcium ionophore ionomycin (2) followed by lysis of the cells with digitonin to obtain R_{max}(3), and finally adding 10mM EGTA(4) and 20mM Tris (5) to obtain R_{min}. **(B)** The curves can then be divided to yield a ratio proportional to the calcium flux in the cell. The essential parts of the trace are marked correspondingly.

Measurements were carried out in a PTI dual emission fluorimeter where the emission of indo-PE3 was simultaneously measured at two wavelengths. Upon adding ethanol, the cells experience a transient elevation in calcium levels (Fig. 3.1 B). The magnitude of the elevation was found to increase with increasing ethanol concentrations but is still very small when compared to antibody stimulation (Figure 3.2). In order to make a better comparison between the responses seen with ethanol and those seen with TcR stimulation, we decided to stimulate the cells with lower concentrations of antibody

in an effort to scale their response down to the level seen with ethanol. We had observed that the maximum response to antibody was obtained when a suspension of Jurkat cells was stimulated with 500ng/mL antibody. By measuring the calcium response from low concentrations of antibody we were able to determine that calcium fluxes obtained from ethanol were comparable in magnitude to elevations obtained from cells stimulated with between 2—8ng/mL antibody (Figure 3.3).

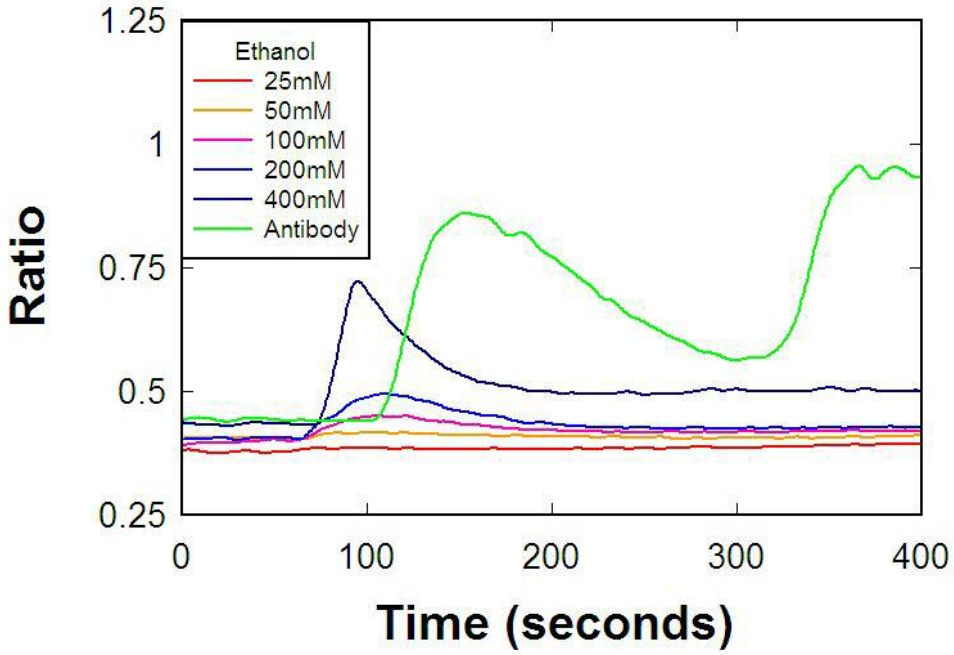

Figure 3.2: Calcium flux increases as the concentration of ethanol increases.

Indo-PE3/AM-loaded cells were stimulated with various concentrations of ethanol and fluorescence ratios corresponding to $[Ca^{2+}]_i$ were calculated from the raw data. As can be seen, the magnitude of the calcium transient was dependent on the concentration of ethanol. The magnitude of ethanol stimulated $[Ca^{2+}]_i$ elevation was typically lower than that seen with TcR stimulation using anti-Vβ-8 Ig followed by a cross linking secondary Ig (marked 'Antibody' in the legend). Of the two $[Ca^{2+}]_i$ peaks with antibody stimulation, the first is seen upon adding the anti Vβ-8 antibody and the second peak is observed upon cross linking with a secondary anti-mouse antibody.

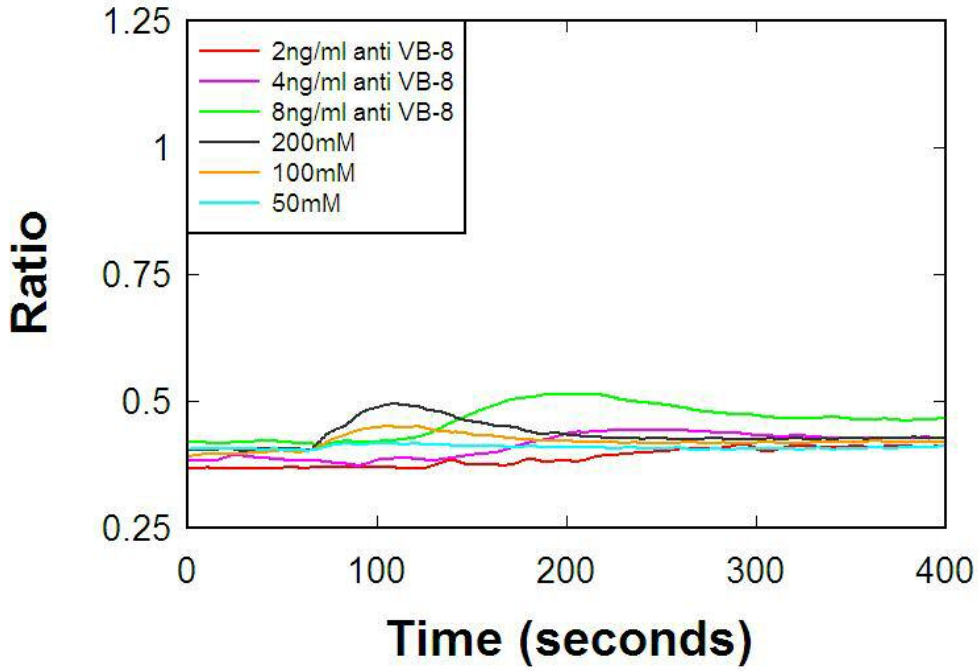

Figure 3.3: Comparison of calcium flux from ethanol and low dose anti-TcR mAb.

Indo-PE3/AM-loaded Jurkat cells were stimulated with low concentrations (2—8 ng/ml) of anti Vβ-8 mAb. The resultant calcium transient was compared to that stimulated by ethanol. From the traces, it appears that 50—200mM ethanol cause calcium fluxes that are roughly comparable to those seen with 2—8ng/ml antibody.

In normal (TcR based) activation of T cells, initial calcium elevation is caused by the IP_3-mediated release of calcium from the intracellular stores in the ER as a result of signaling touched off by TcR stimulation. This results in further elevation of intracellular calcium levels by causing the Calcium Release Activated Calcium (CRAC) channels on the cell surface to open [53, 57]. To determine whether ethanol stimulation also involved the release of calcium from intracellular stores, we repeated the stimulation of Jurkat T lymphocytes in calcium free media containing 100uM EGTA as a chelator of extra-

cellular calcium (Figure 3.4). The initial spike of calcium seen with ethanol stimulation in normal media is largely absent in cells stimulated with ethanol in calcium-free media.

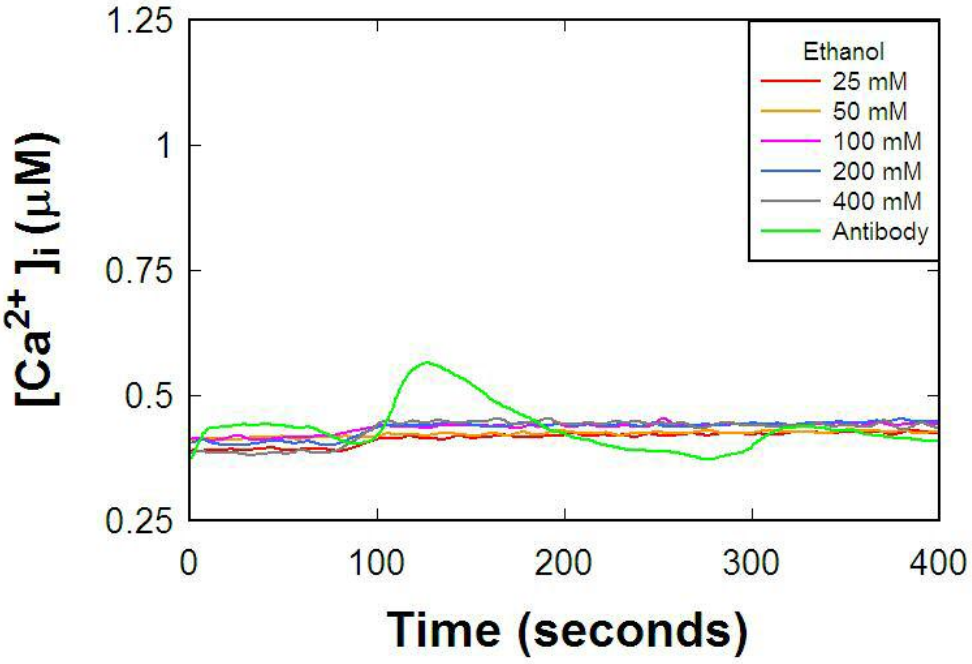

Figure 3.4: Calcium flux in ethanol stimulated cells in calcium free media.

Indo-PE3/AM loaded Jurkat cells were suspended in HBSS containing no added calcium that was supplemented with 1%FBS and 100μM EGTA as a calcium chelator. Upon stimulation with ethanol, the calcium spike that was seen in cells stimulated with ethanol in normal (calcium-containing) medium was absent and calcium elevation in response to ethanol becomes greatly reduced.

The lack of calcium elevation in response to ethanol stimulation in the absence of calcium implied that ethanol triggered the opening of calcium channels. Since CRAC channels are proposed to be the only calcium channel involved in activation of T cells[57], it seemed likely that ethanol was causing these channels to open. Recent studies

have shown that the CRAC channel is actually composed of two closely associated proteins: Stim-1 and Orai-1 [58, 144]. While Orai-1 is thought to be the actual calcium channel, Stim-1, on the surface of the endoplasmic reticulum (ER) is thought to be the activator for Orai-1. The proposed mechanism envisages that calcium release from the ER would cause Stim-1 to bind to Orai-1 and open the calcium channel[145]. Could it be that ethanol somehow causes the opening of the CRAC channels without inducing the release of intracellular stores?

To test the possibility that ethanol was opening the CRAC channels, we incubated Jurkat T cells with the CRAC channel inhibitor 2-aminoethoxydiphenyl borate (2-APB) and then measured cell responses to ethanol. The data show that these cells exhibit no calcium transient in response to ethanol (Figure. 3.5). This was similar to the response seen when Jurkats were stimulated with ethanol in calcium free media. However when 2-APB treated cells were stimulated with antibody, a reduced calcium transient was seen. This difference could be due to the fact that anti-TcR antibody triggers both calcium release (from the ER stores) and calcium influx (via the CRAC channels) whereas ethanol only triggers calcium influx.

As mentioned above, TcR stimulation results in the release of calcium from the ER which is mediated in part by IP_3 binding to receptors on the ER surface[45]. IP_3 itself is generated as a result of signaling events following TcR stimulation that involve Src family kinases. As we have seen in Chapter 2, inhibiting Src family kinases interferes with ethanol mediated adhesion of Jurkat cells to their targets the Raji cells. Interestingly, treating Jurkat cells with PP2 a Src family kinase inhibitor, had no effect on their ethanol stimulated calcium fluxes while it completely eliminated calcium elevation seen with antibody stimulation (Figure 3.6). This suggested that the pathway for elevating calcium differed with different modes of stimulation (ethanol or antibody). Surprisingly, LAT-

deficient ANJ3 cells showed a reduced elevation in response to ethanol (Figure 3.7). LAT is downstream of the Src family kinases in the T cell activation cascade and is vital in transmitting the T cell activation signal downstream [45]. This suggests there are differences in the pathway by which ethanol and antibody trigger calcium elevation.

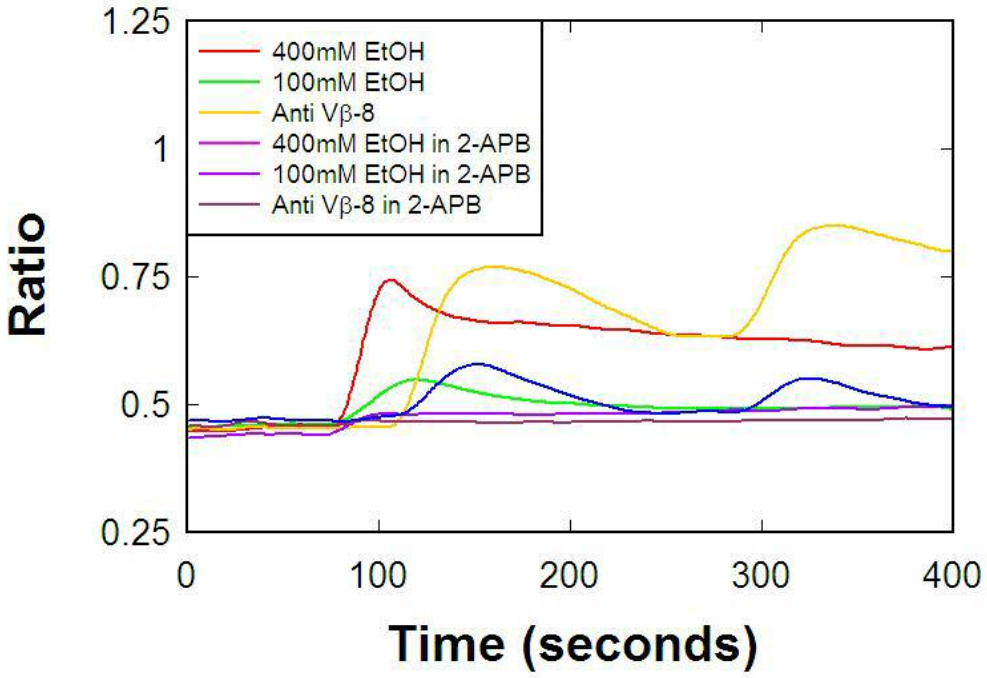

Figure 3.5: 2-APB eliminates ethanol mediated calcium elevation.

Indo-PE3/AM loaded Jurkat cells were incubated with 50μM 2-APB for 10 minutes before being washed and treated with either ethanol or anti-TcR antibody. Stimulation with ethanol resulted in no calcium response whereas antibody stimulation showed reduced calcium transients compared to cells in the absence of the inhibitor.

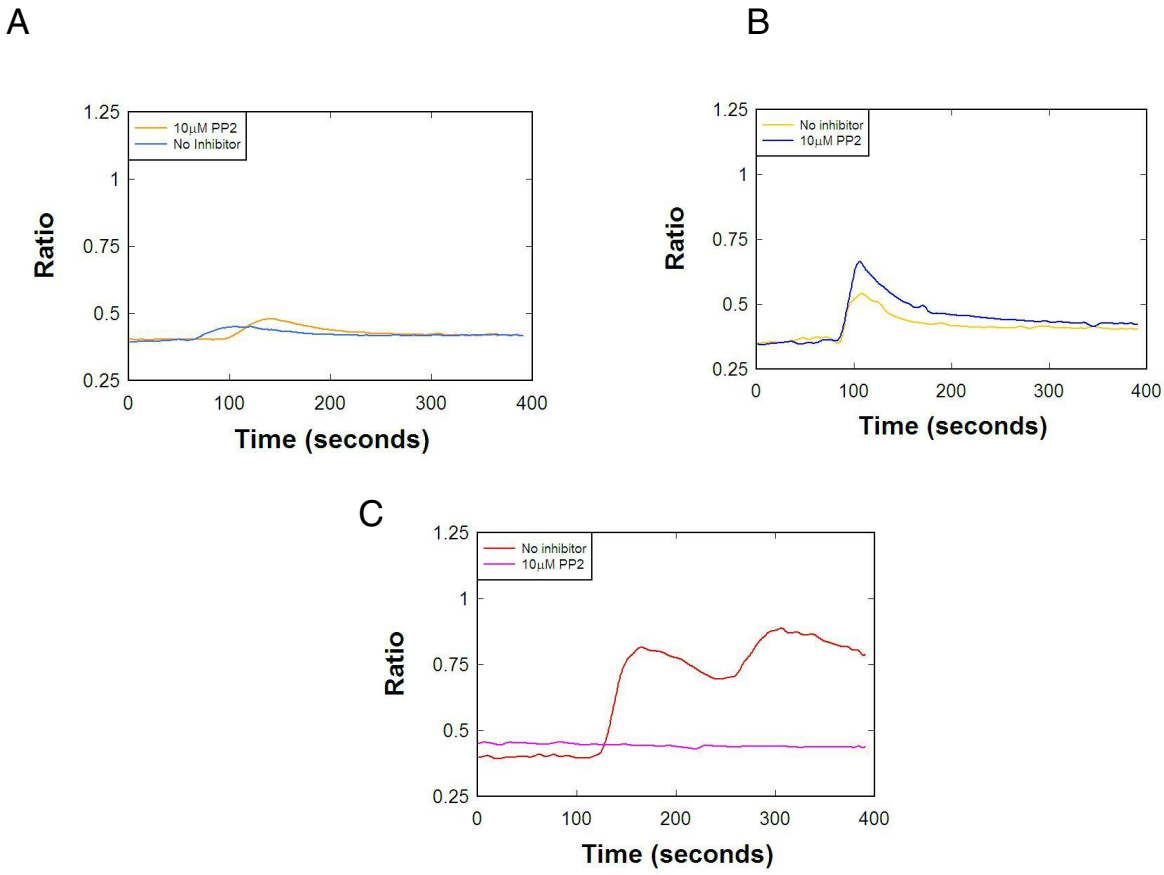

Figure 3.6: PP2 does not inhibit calcium flux triggered by ethanol.

Jurkat cells were treated with 10μM of the Src family kinase inhibitor PP2 before being stimulated with ethanol or antibody. The data show that rather than diminish the elevation of $[Ca^{2+}]_i$ from stimulation with ethanol, PP2 seems to enhance it when cells are treated with (A) 100mM or (B) 400 mM ethanol. (C) In the case of antibody-stimulated cells however, PP2 pretreatment seems to completely eliminate $[Ca^{2+}]_i$ elevation.

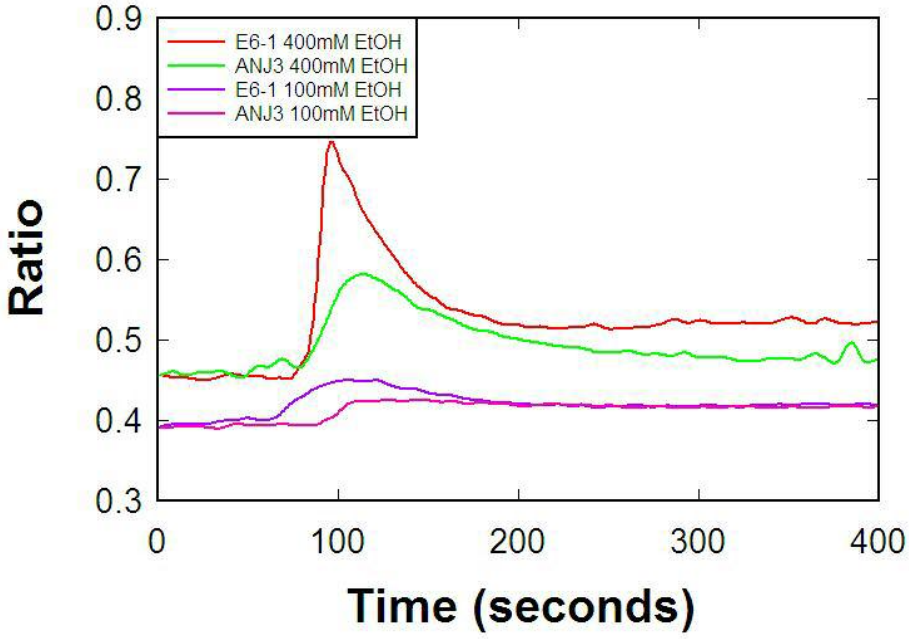

Figure 3.7: ANJ3 cells show reduced calcium response to ethanol stimulation

LAT-deficient ANJ3 Jurkat cells were loaded with Indo-PE3/AM and stimulated with the indicated concentrations of ethanol. The data show that calcium responses from ANJ3 cells are reduced compared to responses from normal E6-1 Jurkat cells.

Given that ethanol may be activating the some of the same signaling cascades as antibody, we decided to look at whether ethanol treatment would have any effect on subsequent stimulation of cells with antibody. Figure 3.8 shows that stimulation with ethanol leads to a modification in the subsequent response to antibody. The extent of this modification depends on the concentration of ethanol used with cells stimulated with 50mM ethanol showing the strongest response to anti TcR mAb whereas cells treated with 400mM show the weakest response.

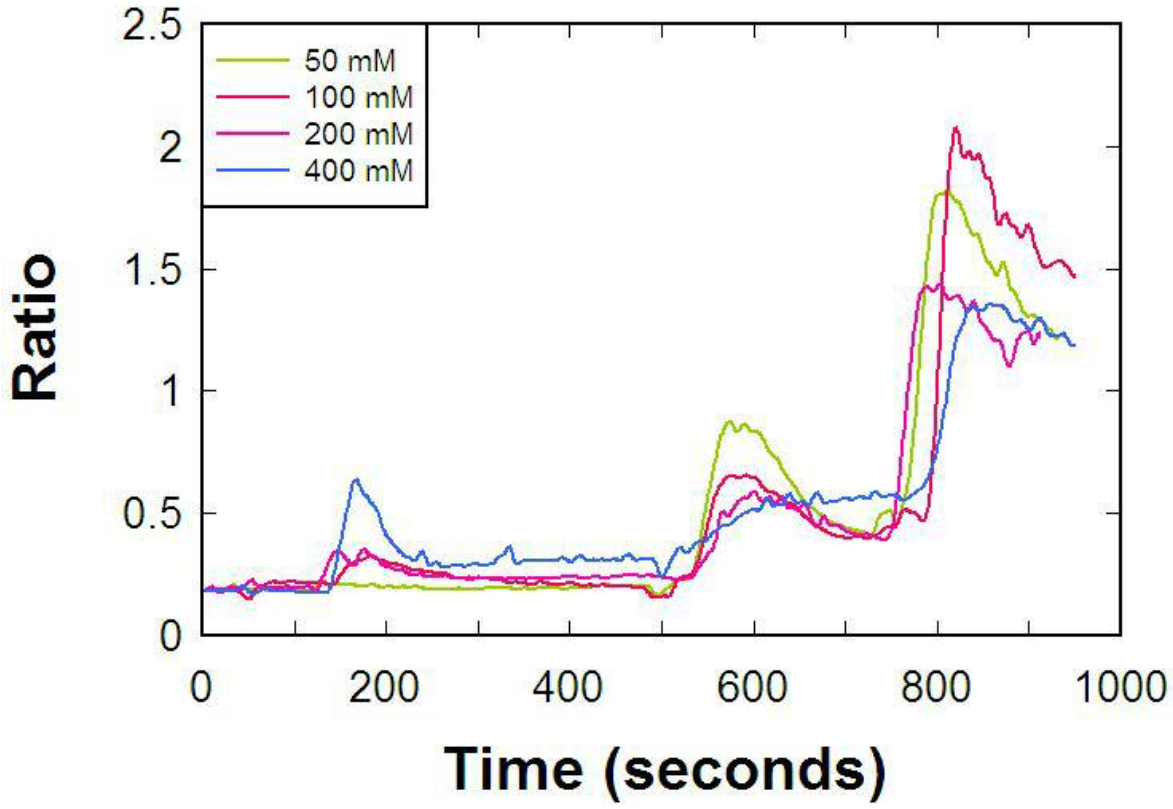

Figure 3.8: Ethanol pretreatment modifies response to subsequent antibody stimulation.

Indo-1 loaded Jurkat cells were stimulated with the indicated concentrations of ethanol followed by subsequent stimulation with anti Vβ-8 mAb and a secondary cross linking antibody (anti mouse). The data show that as cells are stimulated with increasing concentrations of ethanol the elevation in $[Ca^{2+}]_i$ in response to TcR stimulation becomes lower.

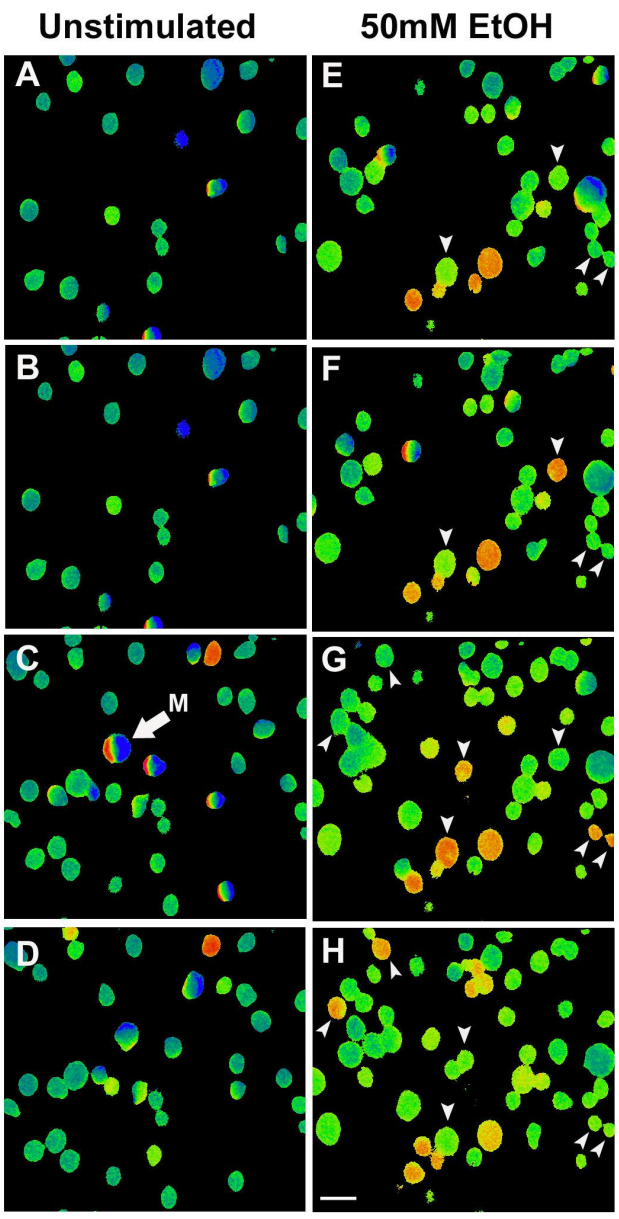

Figure3.9: Ethanol stimulated oscillations in Jurkat cells.

Fluorescent ratio imaging was used to monitor calcium in fura-2 loaded Jurkat cells. **(A—D)** Calcium image data show untreated Jurkat cells are relatively quiescent. Some cells that appear to have elevated calcium are simply movement artifacts (marked M in panel C). **(E—H)** When Jurkat cells are exposed to ethanol (50mM) they exhibit calcium oscillations. Sample images taken at 30 second intervals show calcium levels transiently rise to relatively high levels (>400nM). Arrows mark the oscillating cells. Bar = 10μm.

Calcium flux measurements were repeated using Jurkat T lymphocytes loaded with the Calcium indicator fura-2. These cells were imaged on a fluorescence ratio imaging system consisting of a Zeiss IM-35 microscope coupled to a computer-controlled dual excitation light source and a silicon intensified target (SIT) camera. Specialized

calcium imaging software controlled the acquisition of images at two excitation wavelength, computation of fluorescence ratio images and conversion of the data to intracellular calcium concentration that was displayed as different pseudo-color hues. This technique afforded us the opportunity to study the effects of ethanol on individual cells as opposed to looking at the mean response of a suspension of cells as was the case with indo-PE3 loaded Jurkat cells.

Fura-2 loaded cells were also found to be oscillating in the presence of ethanol (Fig. 3.9). These oscillations were seen rarely in unstimulated cells. Tracing the average calcium levels of these cells over time shows that ethanol-treated cells have an elevated $[Ca^{2+}]_i$ level as compared to unstimulated cells. In the absence of calcium however, the $[Ca^{2+}]_i$ levels in response to ethanol do not increase above resting level (Figure 3.10).

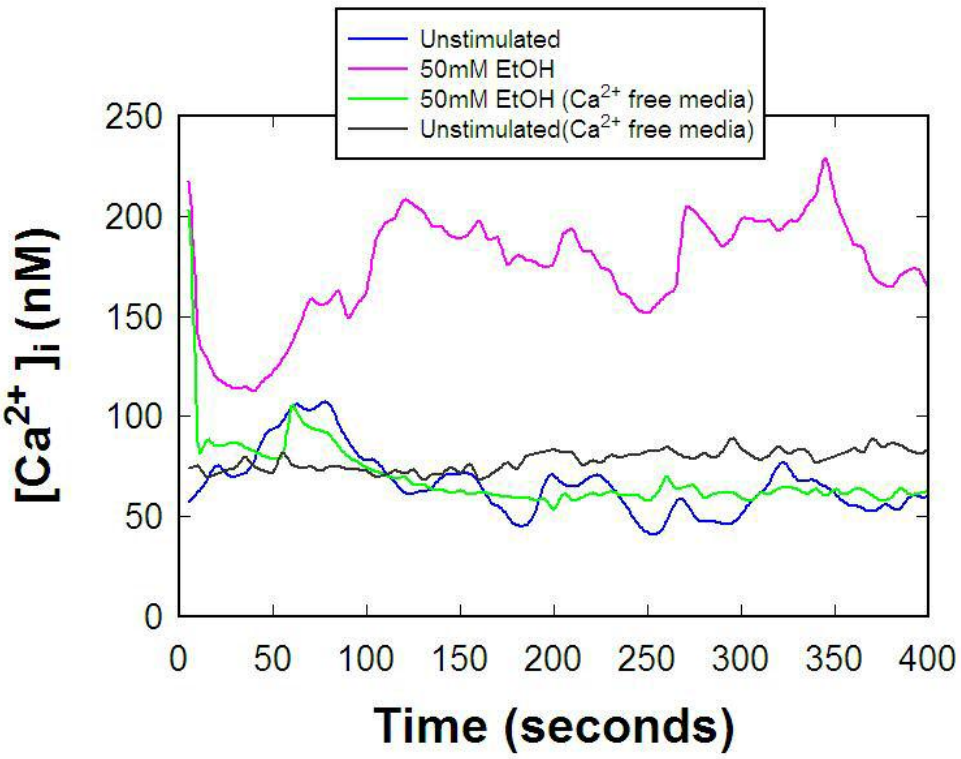

Figure 3.10 Imaging of FURA-2 loaded cells shows elevated $[Ca^{2+}]_i$ in response to ethanol

Traces of average $[Ca^{2+}]_i$ from cells loaded with fura-2 show that ethanol-treated cells have elevated $[Ca^{2+}]_i$ level compared to unstimulated cells. If cells were stimulated in the absence of calcium, they did not show any $[Ca^{2+}]_i$ elevation in line with fluorimeter data.

DISCUSSION

These results demonstrate that stimulation of Jurkat cells by ethanol results in an increase in the $[Ca^{2+}]_i$ which is brought about by the opening of the CRAC channels. The magnitude of ethanol mediated elevation in $[Ca^{2+}]_i$ increases with increasing concentration of ethanol but the elevation is much less than that seen with anti TcR antibody. We found that lower concentrations of antibody (2—8ng/mL) show the same magnitude of $[Ca^{2+}]_i$ elevation as cells treated with 25—100 mM ethanol.

Studies using calcium-free media show that calcium transients due to ethanol are largely due to calcium influx. This is supported by data using the CRAC channel specific inhibitor 2-APB that completely blocks ethanol-stimulated calcium transients. Finally we see induction of calcium oscillations by ethanol. When T cells are stimulated with ethanol, there is an initial large calcium transient followed by sustained calcium oscillations over a period of hours. These oscillations depend on opening of CRAC channels. These observations all indicate that ethanol can trigger opening of CRAC channels.

The oscillations in $[Ca^{2+}]_i$ caused by ethanol stimulation set the stage for inactivation. NFAT is a transcription factor that is activated and translocates to the nucleus as a result of $[Ca^{2+}]_i$ in sufficient frequency[53]. NFAT stimulates both T-cell activation as well as suppression based on the activation of other signaling pathways[70].

We had seen in the previous chapter that ethanol stimulation yields responses that broadly parallel those seen with TcR stimulation. This suggested that ethanol was activating the same signaling cascades in T cells as antigen stimulation. However, in the case of calcium flux, we have found that while the Src family kinase inhibitor PP2 inhibits $[Ca^{2+}]_i$ elevation in response to antibody stimulation, it actually enhances the

calcium flux mediated by ethanol. While LAT-deficient ANJ3 Jurkat cells showed reduced calcium flux in response to ethanol stimulation while $[Ca^{2+}]_i$ elevation in response to antibody stimulation was eliminated. LAT serves as a docking site for PLC-γ which stimulates the release of calcium in normally activated T cells [48].

It should be noted that for studies investigating calcium influx, rather high concentrations of ethanol were used (100 and 400 mM). While these high concentrations of ethanol are not physiologically relevant they allowed us to clearly see the effects of the inhibitors, calcium-free media and mutant cell lines. Since titration of cells with a range of ethanol concentrations showed a continuum in cell calcium response, it seemed likely that the effect of different concentrations of ethanol was one of magnitude rather than qualitative differences in the signaling pathways involved. Furthermore, although the large calcium transient seen at the initial stimulation of T cells with ethanol or antibody is dramatic and does correlate with the strength of the stimulus it is not required for T cell activation. What is critical is the sustained calcium elevation, seen as calcium oscillations that persist long after the initial calcium transient subsides. As we show in the next chapter, long term incubation in low concentrations of ethanol parallels low doses of antibody in terms of prolonged calcium elevation. Thus high concentrations of ethanol are used for studying the mechanism of calcium elevation but low concentrations are used for studying the physiological consequences of ethanol.

Chapter 4 Chronic Ethanol Studies

INTRODUCTION

The response of Jurkat cells to acute treatment of ethanol provided a foundation for understanding the chronic effects of ethanol that might be associated with immuno-suppression. The observation that ethanol elicits responses that broadly parallel those seen with TcR stimulation suggest that ethanol might trigger immunosuppression in a manner that parallels responses to anti-TcR antibody. Anti-TcR treatment in the absence of co-stimulation is one of the most powerful ways to generate tolerance and is administered to patients to prevent graft rejection[146].

Although acute treatment with high concentration of ethanol can blunt the response to subsequent challenges with anti-TcR antibody, the concentration of ethanol needed to see this effect is out of the physiological range. However, we also saw that stimulation with lower concentrations of ethanol (50mM) induced calcium oscillations in the cells (Figure 3.10) and chronic exposure to ethanol might produce similar sustained elevation of calcium. This in turn could set the stage for the activation of transcription factors such as NFAT, which have an important role to play in mediating both proliferative as well as anergic responses of T cells.

In this chapter we investigate the consequences of prolonged ethanol exposure on the stimulation of T cells by antibody. We assay for the effects of chronic ethanol on the T cell signaling pathway as shown by the effects of ethanol in pairing of Jurkats with SEE-coated Raji cells as well as tyrosine phosphorylation of LAT. We also look at how chronic exposure to ethanol affects $[Ca^{2+}]_i$ elevation in response to TcR stimulation with antibody. Finally we look at the translocation of NFAT to the nucleus as a mechanism for

the inhibited response to antibody stimulation seen in cells chronically exposed to ethanol.

MATERIALS AND METHODS

Reagents and Media:

Roswell Park Memorial Institute (RPMI) medium 1640, Iscove's Modified Dulbecco's Medium (IMDM), penicillin-streptomycin, glutamine and sodium pyruvate were purchased from Invitrogen (Carlsbad, CA). Heat inactivated Fetal Bovine Serum (HI-FBS) was obtained from Atlas Biologicals (Fort Collins, CO). Recombinant Human IL-2 was obtained from Chemicon International (Temecula, CA). Partially purified Staphylococcus Enterotoxin E (SEE) was purchased from Toxin Technology (Sarasota, FL). Calcium dyes indo-1 AM and fura-2 AM, the CRAC channel inhibitor, 2-Aminoethyl-diphenyl-borate (2-APB), poly-L-lysine (58 kDa MW), Tween-20, Triton X-100, Sodium Dodecyl Sulfate (SDS), Ethylene diamine tetracetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), and Tris were purchased from Sigma (St. Louis, MO). Indo-PE3 AM and fura-PE3 AM leakage resistant isoforms of indo-1 and fura-2 were obtained from Teflabs (Austin, TX). Criterion nitrocellulose/filter paper sandwiches for immunoblotting were purchased from Biorad (Hercules, CA). Kodak BioMax light film was purchased from Kodak USA (Rochester, NY). Super Signal Pico Chemilumiescent reagent was purchased from Pierce biotechnology (Rockford, IL). Para formaldehyde was purchased from Aldrich (Milwaukee, WI). The nuclear and DNA counter stain 4',6-diamidino-2-phenylindole (DAPI), Cell Tracker Green, Cell Tracker Blue and ProLong® Gold Anti-fade mounting media were purchased from Molecular Probes (Eugene, OR). The Src family kinase inhibitor PP2, 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB or Ellman's reagent) and N-α-benzyloxycarbonyl-L-lysinethiobenzyl ester (BLT) were

purchased from Calbiochem (San Diego, CA). All other reagents were purchased from Sigma (St. Louis, MO).

Antibodies:

Mouse monoclonal anti CD3, anti Vβ-8, anti NFAT-1, anti PLC-γ, anti PKC-θ and anti-ADAP mAbs were purchased from BD-Pharmingen (San Diego, CA). Mouse Anti phospho-LAT mAb was purchased from Cell Signaling Technology (Danvers, MA). Rabbit anti LAT was purchased from Upstate (Charlottesville, VA). Rat anti α-tubulin was purchased from Chemicon International (Temecula, CA), while mouse anti β-tubulin and Cy-3 conjugated rabbit anti γ-tubulin were from Sigma (St. Louis, MO). All fluorescently conjugated anti mouse and anti rabbit antibodies were purchased from Jackson Immuno laboratories (West Grove, PA).

Cell Lines:

Jurkat (clone E6-1) human peripheral blood leukemia helper T cell line, Raji human Burkitt's lymphoma B cell line, and TALL 104 human acute lymphoblastic leukemia cytotoxic T cell line were obtained from ATCC (Manassas, VA). LAT deficient Jurkat cells (ANJ3) were a generous gift from Dr. Lawrence E. Samelson (National Cancer Institute at the National Institutes of Health). LFA-1 deficient Jβ-2.7 Jurkat cells were a generous gift from Dr. Timothy Springer (Department of Pathology, Harvard Medical School).

Cell Culture:

Jurkat and Raji cells were cultured in RPMI medium 1640 containing 10% HI-FBS, 50 μM β-mercaptoethanol, 24 mM NaHCO3, 1 mM pyruvate, and 1 mM glutamine. TALL 104 cells were cultured in IMDM containing 20% FBS and 100 units/μL

recombinant human IL-2. All cells were grown in a 37°C incubator with 5% CO2. Media was replenished every two to three days.

Preparation of Poly-L-lysine coated coverslips:

No. 1 Glass coverslips (20 X 20 cm) were immersed in a 10% (v/v) solution of KOH (from a saturated stock solution) in Ethanol for 1 hr. The coverslips were washed by repeated immersion in distilled/de-ionized water (dd-H_2O). Excess water was wicked off on a paper towel and a small amount (25µL) of a 50mg/mL solution of 56KDa poly L-Lysine was applied to the cover slip and gently spread using a clean glass Pasteur pipette. The coated cover slip was washed again; excess water wicked off with a paper towel, covered to prevent any dust from settling and air dried at room temperature. Poly-Lysine coated coverslips were always freshly prepared a day ahead of an experiment. The dried coverslips were then each placed in a well of a six well plate where the cells were plated, fixed and immuno stained.

Conjugation of Jurkat and Raji cells

Raji cells were incubated with 2µg/mL SEE for 30—45 minutes. These cells were also labeled with Cell Tracker Blue as follows. Raji cells were incubated with 1µM Cell Tracker Blue and incubated for 15 minutes at 37^{o}C, 5% CO_2.The Raji cells were then washed twice resuspended at 1 X 10^7 cells/ml in RPMI with 1% FBS, mixed with an equivalent amount of Jurkat cells, plated on coverslips and incubated for 15 minutes at 37^{o}C, 5% CO_2 to allow conjugation.

Preparation of Fixative

1 gram of parafomaldehyde was de-polymerized by boiling in 10mL dd-H_2O in the presence of 50uL 2N NaOH solution to give a 10% w/v formaldehyde solution. The

formaldehyde solution was then diluted to 3.7% in PBS containing 1mM Calcium and 5mM Glucose.

Immuno-staining

Coverslips with adhered cells were then fixed in this solution for 30 minutes at room temperature. The cells were briefly washed thrice in PBS and permeablized with ice cold 1:1::Acetone:Methanol and incubated on ice for 15 minutes. The cells were then washed thrice for 5 minutes each on an orbital shaker at 75 RPM with PBS and blocked for 30 minutes with 5% goat serum, 0.1% Tween-20 in PBS. The cells were once again washed thrice for 5 minutes each on an orbital shaker. The cells were then incubated with the primary antibodies for one hour. Subsequently, the cells were washed 6 times (5 minutes each on orbital shaker) in PBS, incubated with the secondary antibody, washed again as before [51], and mounted on glass slides using the Prolong® Gold mounting media[114].

Image Acquisition and Processing

Images were acquired using a Nikon Diaphot 200 Inverted microscope using a Hamamatsu Orca CCD camera (Hamamatsu Corp., Bridgewater, NJ). Z-axis stacks consisting of 256 successive images were acquired using a MAC 2000 Z-axis focus controller (Ludl Electronic Products, Hawthorne, NY) and a custom image acquisition plugin written for ImageJ [51]. Point spread functions for fluorescein and rhodamine fluorophores were generated using 0.2 micron fluorescent micro spheres obtained from Molecular Probes (Eugene, OR) [114]. Images were deconvolved using the maximum likelihood algorithm of XCOSM adapted to run on a PC [115].

Cell stimulation and Immunoblotting

E6-1 Jurkats cultured chronically in various concentrations of ethanol were counted washed and resuspended at 5 X 10^6 cells/ml. 1 ml aliquots of cells in 1.5 ml centrifuge tubes were either left unstimulated or stimulated with 0.5 μg of anti Vβ-8 mAb, incubated for 1 minute, then treated with 0.5 μg of an unconjugated goat anti mouse Ab and incubated for 1 more minute at room temperature.

Following incubation, the cells were pelletted in the cold (4^{o}C) in a VWR desktop microfuge. The supernatant was removed by aspiration as the cells were lysed immediately in 400uL of hot 2X sample buffer (20 mM Tris (pH8.0), 2 mM EDTA, 2 mM Na_3VO_4, 20 mM DTT, 2% SDS, and 20% glycerol) [116]. The samples were then homogenized by passing through a 21-ga syringe needle, heated in a boiling water bath for 5 minutes and cleared by centrifugation at 250 X g.

Proteins were resolved by SDS-PAGE, then transferred to a 0.2μm nitrocellulose membrane. The blot was blocked in 5% non-fat powdered milk solution in Tween-20 containing Tris Buffered Saline (T-TBS) (25 mM Tris/137 mM NaCl/2.7 mM KCl/0.025% Tween-20, pH 7.4). The membrane was incubated with agitation for 1 hr with the appropriate primary antibody diluted in blocking solution and subsequently washed thrice in T-TBS for 10 minutes each with agitation. The membrane was then incubated with a Horse Radish Peroxidase (HRP) conjugated secondary antibody for 1 hour before being washed thrice in T-TBS for 10 minutes each with agitation. The blots were developed using the Pierce Super Signal Pico Chemilumiescent development kit and detected by exposure to Kodak Biomax HR film.

Calcium Measurements

Fluorimeter measurements

E6-1 Jurkats were counted, washed and resuspended at a concentration of 1 X 10^6 cells/ml in pre-warmed HBSS with 25mM HEPES buffer, 5mM Glucose, 1% FBS, pH 7.2, and loaded with 1 μM indo-PE3 for 1.5—2 hours at 37°C. The cells were then washed once with HBSS, resuspended at a concentration of 1 X 10^6 cells/ml and incubated at 37°C for 10 minutes to allow complete de-esterification of the dye inside the cells. Measurements were carried out in a PTI dual emission fluorimeter in a standard fluorimeter cuvette using 3ml aliquots of cells.

The dye was excited at 360nm with emitted fluorescent light monitored by two photomultiplier tubes at 404 and 485nm. The ratio of the 404 and 485nm signals provides a measure of the $[Ca^{2+}]_i$ [138]. The cells were kept in constant agitation using a magnetic stirrer. The resting $[Ca^{2+}]_i$ of cells was recorded, the cells were then stirred by pipetting up and down using a transfer pipette and $[Ca^{2+}]_i$ recorded again. The stimulant (ethanol or antibody) were then added, the cells and stirred and $[Ca^{2+}]_i$ was recorded for 400 seconds. To calibrate the signals we obtained the maximum (R_{max}) and minimum (R_{min}) values for the calcium ratio. To obtain R_{max}, 2μM of the calcium ionophore ionomycin was added to the cells. The cells were then lysed with digitonin to ensure saturation of the dye with Ca^{2+} present in HBSS. In order to obtain R_{min}, 10mM EGTA and 20mM Tris were added to the lysed cells. These calibration steps were done at the end of each run.

Imaging measurements:

The cells were counted, washed and resuspended at a concentration of 1 X 10^6 cells/ml in pre-warmed HBSS with 25mM HEPES buffer, 5mM Glucose, 1% FBS, pH 6.8 and loaded with 2μM fura-PE3 AM for 1 hr at 37°C. For loading with fura-2 AM, the

procedure was the same except that the pH was 7.2 and the cells were incubated with the dye for 30 minutes[139]. Cells were then pelleted and resuspended in HBSS.

The cells were then added to a 22 mM square poly-L-lysine coated cover slip attached using silicon vacuum grease to the bottom of a 35mm diameter Petri dish containing a hole about 15mm in diameter and allowed to adhere. The cover slip and its mount were then placed in an aluminum water jacketed holder. The holder was mounted on the stage of a Zeiss IM-35 microscope. The temperature was held constant at 37°C by circulating heated water through the cover slip holder and through coiled tubes circling the Nikon UV-F 40X/1.3NA objective lens.

Fluorescence ratio images were collected and processed essentially by the same process as described by Poenie and Tsien[140]. A PTI Deltascan dual monochromator light source was used to generate dual excitation wavelengths. Images were processed using the PTI Image Master system. Images were calibrated by dividing each raw ratio image by the ratio image obtained at the beginning of each experiment from a thin film of solution containing 25 μM fura-2 penta-potassium salt in 10 mM EGTA, 50mM HEPES, pH7.2. The resulting ratio images were expressed as a multiple of Rmin. For calculations of [Ca2+]i, Rmax was pre determined as described[141] and the equation (R-Rmin)/(Rmax-R) x K was used with K being a predetermined constant for the dye. Calcium levels are coded as pseudo-colored hues ranging from blue, which represents low calcium values to purple, which represents the high calcium levels. [Ca2+]i values were extracted from pseudo color images using NIH Image J software with PTI acquire image and multi-measure plug-in.[142].

RESULTS

To test the long term effects of alcohol on calcium levels, Jurkat cells were cultured in the presence or absence of ethanol or anti-vβ-8 over a period of 96 hours. Subsequently cells were loaded with indo-PE3 and then tested for their response to anti-CD3 antibody (Figure 4.1). The results show that before treatment with anti-CD3 Ig, the calcium baseline of cells treated with ethanol or antibody was already markedly elevated compared to untreated cells. When these cells were challenged with anti-CD3, the response was severely dampened.

The elevated calcium baseline in cells exposed to ethanol for 96 hours seen in figure 4.1 could be due to a mixed population of cells where some had very high levels of calcium and some were very low levels, typical of normal cells. Alternatively, the population might be more uniformly elevated. To distinguish between these possibilities we imaged calcium levels in individual cells using fura-2. The results showed that the population of cells showed elevation of calcium (Figure 4.2).

To determine whether the ethanol-mediated suppression of response to antibody stimulation was reversible or not, jurkat cells were cultured in ethanol for 96 hours and then transferred to ethanol free media. Within 2 hours of removing ethanol, chronically treated cells started to show improved $[Ca^{2+}]_i$ in response to anti TcR stimulation (Figure 4.3). By 24 hours after removal of ethanol, cells showed nearly complete recovery in both baseline calcium levels and near normal responses to stimulation with anti-CD3.

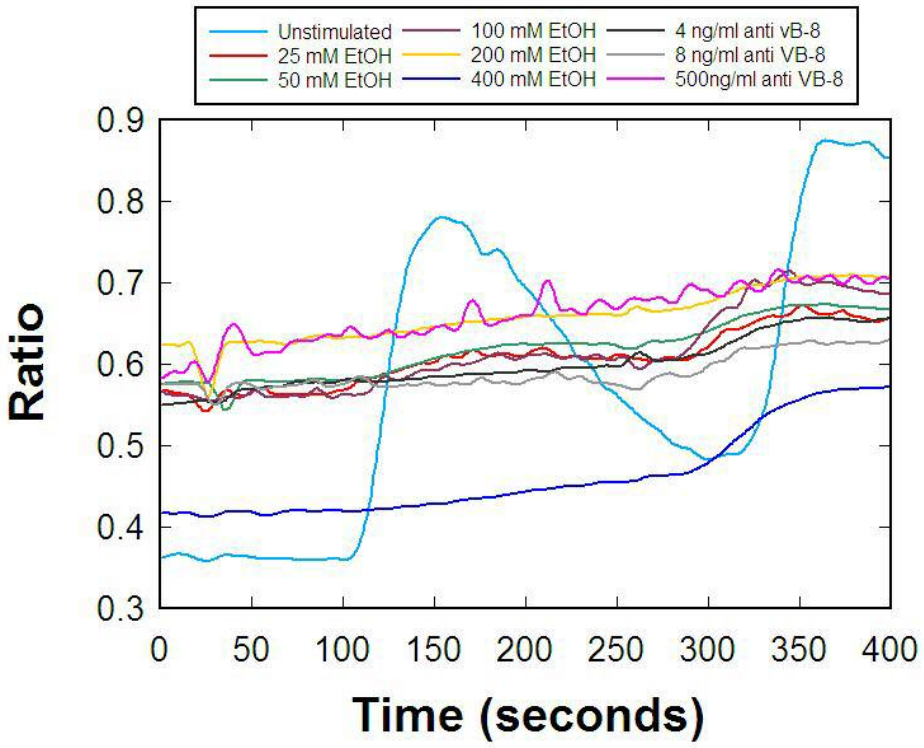

Figure 4.1: Chronic ethanol treatment diminishes calcium signaling in response to anti TcR Ig.

Jurkat cells were incubated in various concentrations of ethanol or anti-Vβ-8 for 96 hours. They were then loaded with indo-PE3/AM and stimulated with anti-CD3 mAb followed by a secondary anti-mouse Ig. The results show that chronic ethanol treatment causes elevation of baseline calcium and greatly reduces the response of cells to subsequent treatment with anti-CD3 mAb although some response is still detectable when the secondary anti-mouse Ig is added. Note that chronic treatment with anti-vb8 Ig down to 4ng/ml or ethanol down to 25mM gave similar results.

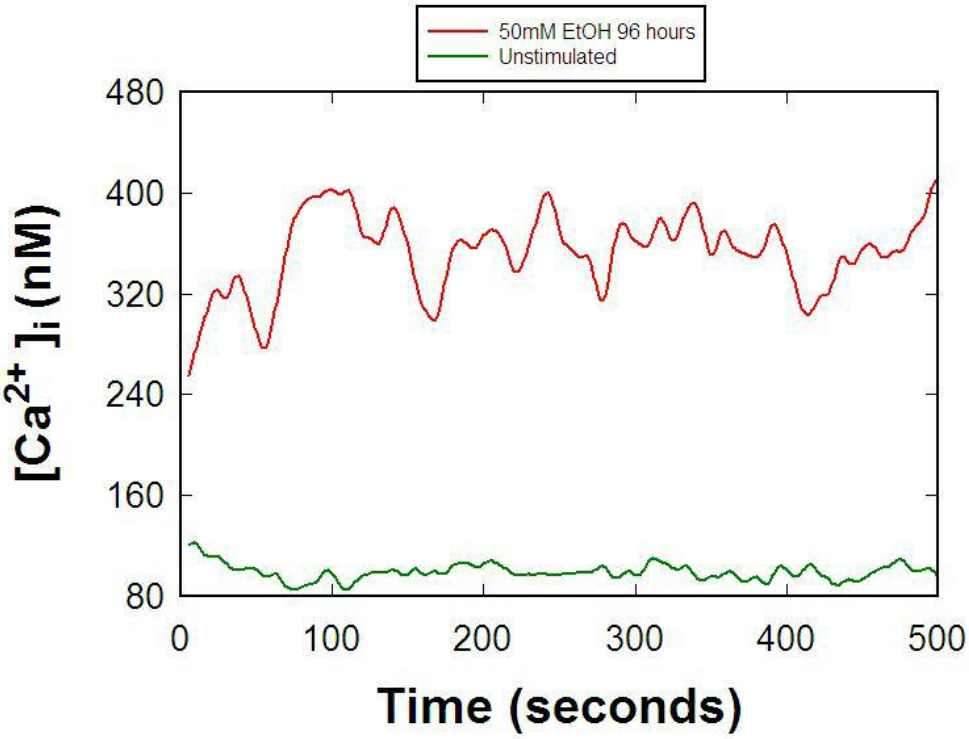

Figure 4.2: Chronic ethanol treatment causes calcium oscillations at higher baseline.

Jurkat cells were incubated with 50mM ethanol for 96 hours, then loaded with fura-2 and imaged. Chronic ethanol treatment leads to cells having a higher $[Ca^{2+}]_i$ baseline similar to what was observed with fluorimetry. Continued oscillations in cells may be the cause for elevated baseline levels of $[Ca^{2+}]_i$

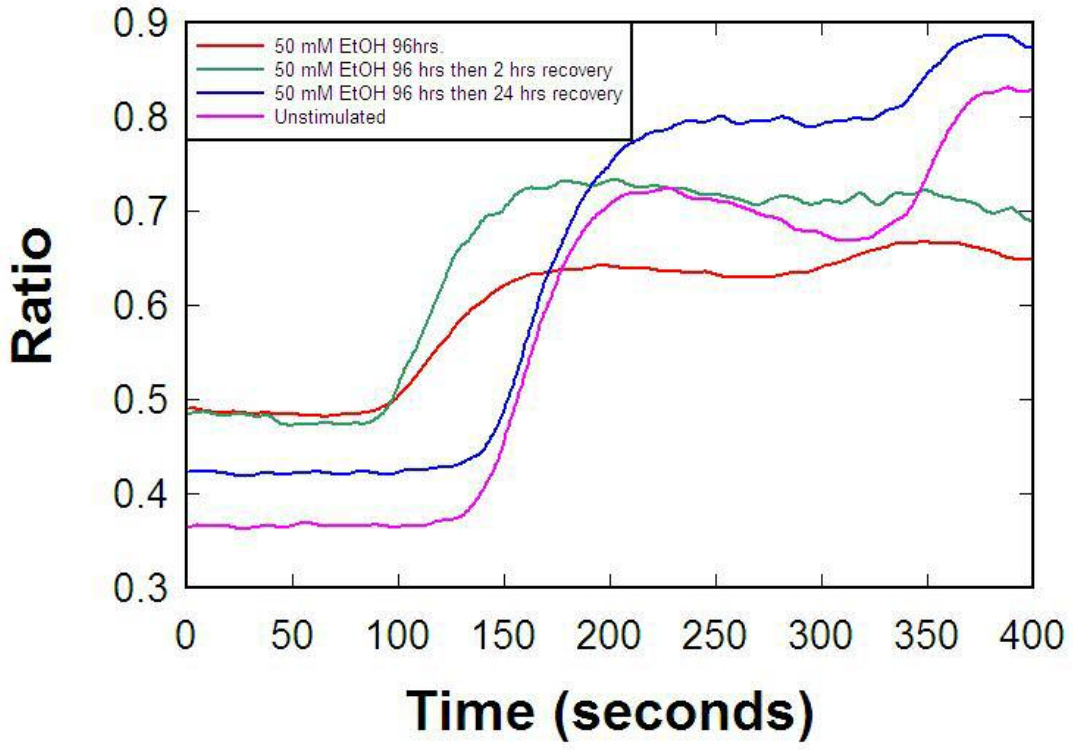

Figure 4.3: Recovery of antibody triggered calcium signaling responses after the removal of ethanol

Jurkat cells were incubated in 50mM ethanol for 96 hours and then were transferred to ethanol free media for the indicated amounts of time. The chronically treated cells started to show increased response to antibody stimulation 2 hours after removal of ethanol. By 24 hours after the removal of ethanol, the cells had returned to a nearly normal baseline and were also showing a nearly normal response to antibody stimulation.

Sustained elevation of $[Ca^{2+}]_i$ is harmful for cells and leads to apoptosis. Exposure to ethanol has been reported to induce apoptosis in thymocytes[147, 148]. These reports suggest that ethanol induced apoptosis was the result of an increase in the $[Ca^{2+}]_i$ levels in cells. We have already seen that chronic exposure to ethanol not only causes a sustained elevation in the $[Ca^{2+}]_i$ levels, but it also leads to the inhibition of antibody stimulation of cells. We wanted to know if this inhibition was caused in part by cell death due to prolonged exposure to ethanol. Since ethanol leads to elevated $[Ca^{2+}]_i$, we were also interested in determining if removing calcium would help ameliorate any ethanol induced cell death.

In this regard we cultured jurkat cells in ethanol for 96 hours in the presence and absence of ethanol and then determined the number of living and dead cells using Trypan Blue exclusion [149, 150]. We found that ethanol does not cause any significant cell death at lower (50 and 100mM) concentrations of ethanol (figure 4.4). Ethanol treatment did result in lower number of viable cells however and in the absence of significant number of dead cells, this implied that ethanol treatment would result in inhibiting or slowing down the proliferation of cells. It is pertinent to note here that one of the characteristics of immuno-suppression is the propensity of T cells to become hypo-reactive as well as enter a senescent phase.

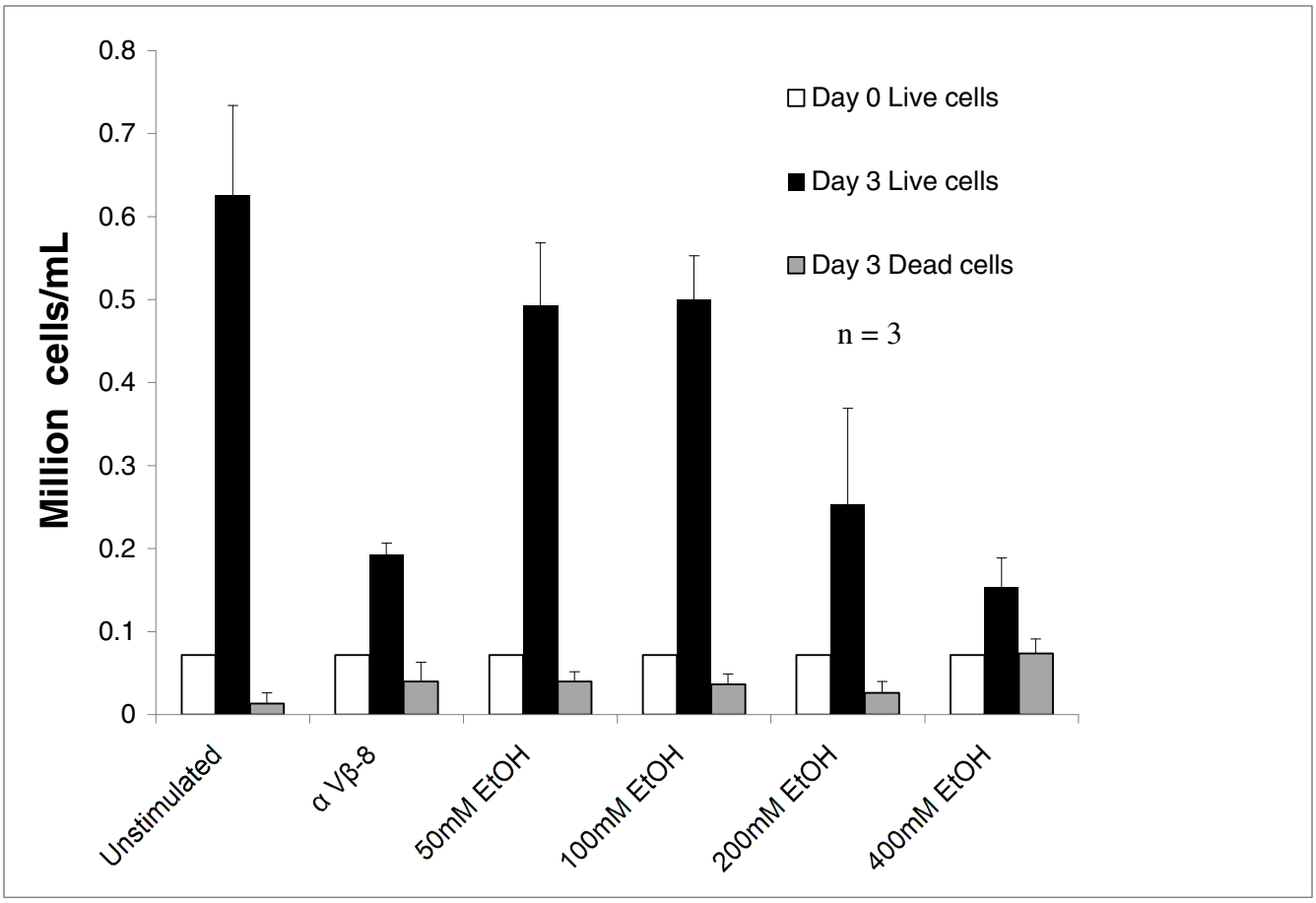

Figure 4.4: Chronic ethanol exposure does not cause significant loss of T cells.

Jurkat cells were cultured in RPMI 1640 containing 10% FBS and chronically exposed to ethanol or antibody. Chronic exposure to low concentrations(50 and 100mM) of ethanol did not result in significant death of cells as assessed by Trypan Blue exclusion. The total number of viable cells was reduced in ethanol and antibody treatments which suggested an overall suppression of proliferation. Error bars show standard error where n is the number of independently repeated experiments.

Since lower concentrations of ethanol did not cause large amounts of die-off, we focused our attention on NFAT. Previous studies have shown that when NFAT is activated apart from other transcription factors associated with a normal activation program (AP-1 and NFκB) transcription is diverted to expression of inhibitory factors such as E3 ligases that break down PLC-γ and PKC-θ [54]. NFAT is activated through

the calcium-dependent phosphatase calcineurin. Calcineurin dephosphorylates NFAT exposing its nuclear localization sequence resulting in its translocation to the nucleus[70]. To determine whether ethanol could cause NFAT translocation, we used immuno-staining to monitor the location of NFAT in cells.

Cells were incubated in 50 mM ethanol for various intervals of time (24, 48 and 72 hours) before fixing and staining for NFAT. We saw NFAT localizing in the nucleus at 24 hours onwards (Figure 4.5). Such movements were not seen in unstimulated cells.

Since anergy reportedly depends on the activation of NFAT apart from other transcription factors [54], we also monitored the location of NFκB by immunostaining. Jurkat cells were exposed to 50mM ethanol for 24, 48 and 72 hours and immunostained for NFκB. The data shows that NFκB does not translocate to the nucleus under these conditions (Figure 4.6).

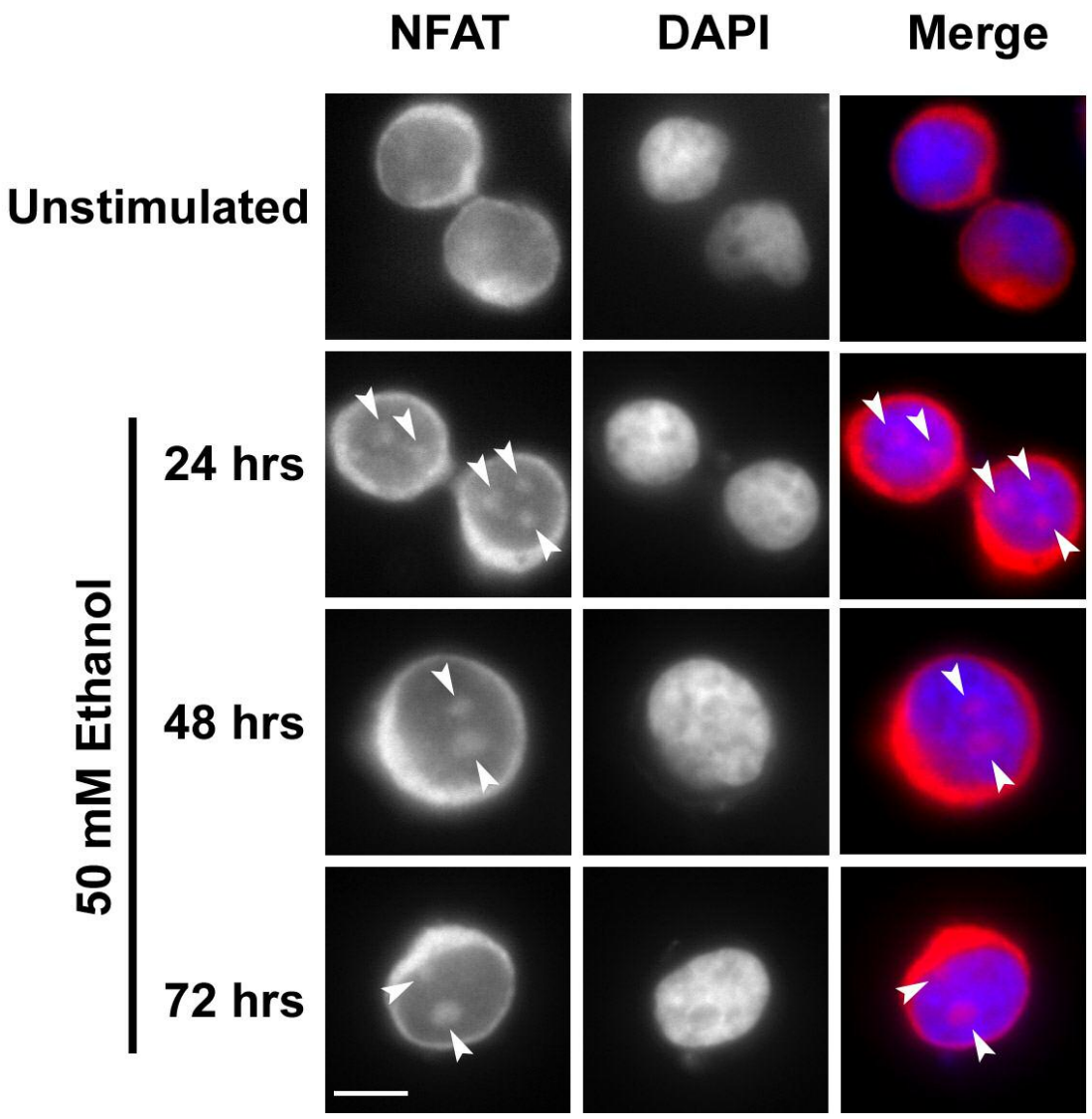

Figure4.5: Chronic ethanol causes NFAT translocation to the nucleus.

Jurkat cells were cultured in 50mM ethanol for the indicated periods of time, after which they were fixed and immuno-stained for NFAT. The nuclear stain DAPI was used to counter-stain the nucleus in these cells. While there is no NFAT evident in the nuclei of unstimulated cells, exposure to ethanol causes NFAT to translocate to the nucleus within 24 hours. NFAT remains in the nucleus for longer periods of time in the presence of ethanol. Bar 5µM.

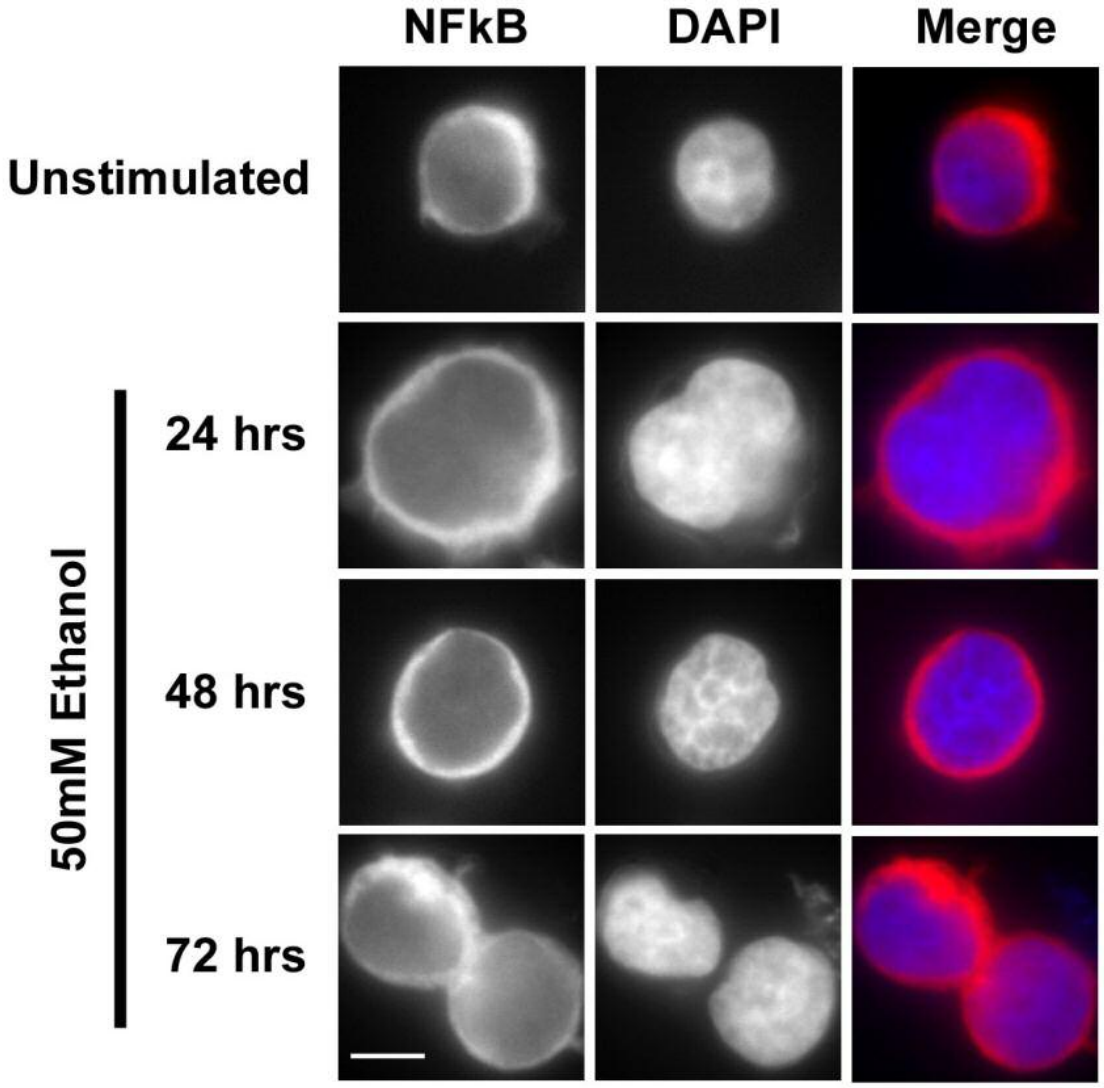

Figure 4.6: Chronic ethanol does not lead to NFκB translocation into the nucleus

Jurkat cells were cultured in 50mM ethanol for the indicated periods of time and then fixed and immunostained for NFκB. The nuclear stain DAPI was used to counter-stain the nucleus in these cells. As can be seen, there is no evidence of NFκB translocation to nuclei of unstimulated or ethanol-treated cells. Bar 5μM.

The observation that chronic ethanol exposure causes the inhibition of $[Ca^{2+}]_i$ elevation in response to antibody stimulation as well as causing the translocation of NFAT but not NFκB into the nucleus, suggests that ethanol may be causing Jurkat cells to become nonresponsive. So far we have only seen chronic ethanol reduced the elevation of [Ca2+]i in response to antibody-stimulation, but we have not determined whether chronic ethanol has any effect on other functional aspects of T cell activation. Moreover, as mentioned before, in an inhibitory transcription program, NFAT causes the expression of E3 ligases which in turn target enzymes vital to the T cell activation pathway such as PKC-θ and PLC-γ for destruction. In this regard we stimulated cells chronically exposed to ethanol with anti TcR antibody lysed and immuno-blotted the lysates to determine whether there was any effect on phosphorylation of LAT in response to antibody stimulation as well as the levels of PKC-θ and PLC-γ. Preliminary data in Figure 4.7 shows that chronic stimulation of cells with antibody inhibits LAT phosphorylation in response to antibody stimulation. However, in cells exposed to ethanol for 96 hours, the reduction in phospho-LAT seems to stem from a reduction in the endogenous levels of the protein in the cells. Interestingly, chronic ethanol exposure has no effect on the levels of endogenous PLC-γ.

LAT is an adaptor protein that is vital to the propagation of the T cell activation signal. Lowering of LAT phosphorylation in response to antibody stimulation implied that chronic exposure to ethanol was inhibiting the normal T cell activation.

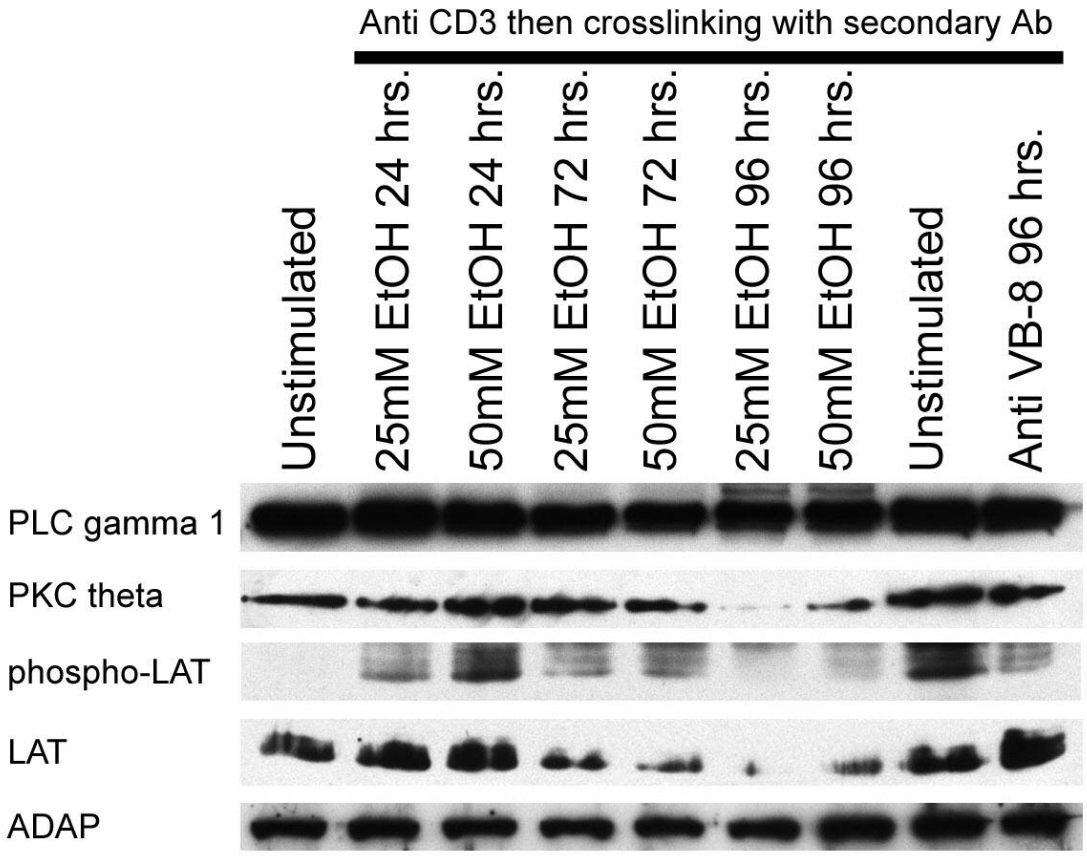

Figure 4.7: Chronic ethanol exposure leads to reduced levels of LAT.

Jurkat cells were cultured in 25 and 50mM ethanol or anti-Vβ-8 antibody for the indicated times and then were challenged with anti-CD3 antibody followed by cross linking with a secondary antibody. The cells were then lysed and immunoblotted for phospho-LAT, PKC-θ and PLC-γ. As can be seen, there was a visible reduction in the LAT phosphorylation levels which seems to arise from the reduction in the levels of endogenous LAT in the cells as the period of chronic exposure to ethanol prolonged. However there was no significant change seen in PLC-γ levels. ADAP is used as loading control.

DISCUSSION

Prolonged exposure to ethanol has been shown to cause immunosuppression[78, 151]. Our studies show that exposure to ethanol for 96 hours leads to elevated basal $[Ca^{2+}]_i$ levels followed by loss of the $[Ca^{2+}]_i$ transient seen when cells are stimulated with anti-CD3 Ig. Once ethanol is removed however, both baseline $[Ca^{2+}]_i$ and the response to anti-CD3 starts to recover within 2 hours and return to normal levels after calcium is removed. Similar patterns of elevated baseline calcium levels and loss of signaling responses are seen when cells are cultured in low doses of antibody. The loss of response to antibody coincides with reduced expression of the adapter protein LAT .and reduced adhesion to SEE-coated Raji cells as compared to unstimulated Jurkat cells.

There are reports that calcium along with other signaling pathways leads to productive activation while calcium by itself leads to decreased responsiveness and subsequent anergy[152]. One of the ways in which elevated $[Ca^{2+}]_i$ leads to anergy is through the activation of NFAT. In cooperation with other transcription factors such as AP-1 and NFκB, NFAT transcribes genes involved in a productive immune response[153]. However in the absence of other transcription factors, NFAT turns on a T-cell anergy program by transcribing genes for E3 ligases that target signaling proteins such as PKC-θ and PLC-γ. We observed nuclear accumulation of NFAT but not NFκB, in Jurkat cells incubated for 96 hours in ethanol. Upon immunoblotting lysates from cells that were chronically treated with ethanol and then stimulated with antibody, we found that tyrosine phosphorylation of LAT in these cells was reduced. There was no effect on the expression of PLC-γ. There does however appear to be a reduction in the levels of PKC-θ as well as endogenous LAT.

Chapter 5: Conclusion

ETHANOL ACTIVATION AND SUPPRESSION OF T CELLS:

The results presented above lay down a framework for explaining how acute ethanol could cause immunosuppression. This model suggests that activation of T cells by ethanol could trigger anergy in a manner similar to that of other incomplete stimuli such as when T cells are activated by antibody alone.

There are many lines of evidence showing that ethanol activates Jurkat cells. The first of these is the observation that ethanol induces adhesion between Jurkat and Raji cells. These adhesions are dose-dependent, peaking at 25 mM ethanol before trailing off at higher concentrations. Adhesions are also dependant on the integrin LFA-1, the adapter protein LAT and Src family kinases as evidenced by the loss of adhesion using LFA-1 and LAT deficient Jurkat cells or when normal Jurkat cells are treated with the Src family kinase inhibitor PP2.

Integrin mediated adhesion between T cells and targets results from signaling events inside the cells that lead to the active form of integrin and increased affinity for and avidity with its receptor on the target cell. In normally activated cells, LFA-1 clusters in the shape of a ring at the immune synapse in what is known as the pSMAC. The pSMAC also consists of F-actin and the adapter protein ADAP. ADAP has recently been shown to be involved in anchoring the microtubule motor protein cytoplasmic dynein, thus playing an vital role in the polarization of the MTOC to the contact site and the release of effector molecules[114]. In ethanol stimulated synapses, we see not only LFA-1 but also the adaptor protein ADAP as well as F-actin clustered into the shape of a ring. Moreover, a significant portion of Jurkat cells in ethanol mediated pairs had their MTOCs polarized to the synapse.

In addition to synapse formation we also obtained physiological and biochemical evidence for T cell activation by ethanol. For example, we observed that ethanol induced the tyrosine phosphorylation of the adapter protein LAT. We also showed that ethanol induced the release of cytolytic granzymes as measure by an increase in BLT esterase activity.

At present, we do not know how ethanol induces LAT phosphorylation. LAT is normally phosphorylated by ZAP-70 which itself is phosphorylated and activated by the Src family kinase Lck[47]. Since both these proteins are phosphorylated in their active state, it should have been possible to detect an increase in activity based on an increase in phosphorylation. However, immunoblotting of lysates from ethanol stimulated Jurkat cells showed no evidence of phosphorylation and activation of either Lck or ZAP-70. Another possibility is that LAT phosphorylation might be dependent on Fyn. Some reports have suggested that LAT activation does not require Fyn[154] however a more recent study has reported diminished LAT phosphorylation when Fyn deficient naïve CD4+ T cells are stimulated with anti-TcR antibody[155].

The possible involvement of Fyn in ethanol-induced tyrosine phosphorylation is interesting because of known effects of ethanol on neuronal Fyn. In mice it was found that ethanol induced Fyn to phosphorylate the N-methyl-D-aspartate (NMDA) receptor in neurons [156]. The NMDA receptor is a postsynaptic ion channel that opens in response to binding glutamate (also know as a ionotropic glutamate receptor) [157]. Activation of the NMDA receptor opens an ion channel that is non selective for cations. This pore allows the flow of Calcium and Sodium into the cell and Potassium out of the cell. Calcium flux through the NMDA receptor is thought to play a critical role in synaptic plasticity [158]. In response to phosphorylation by Fyn, the NMDA receptor function is modulated resulting in tolerance to acute ethanol exposure[156].Deletion of the Fyn gene

results in increased behavioral sensitivity to ethanol [159] while over-expression reduces sensitivity to ethanol [160]. Given the ability of ethanol to activate Fyn in neurons, it is possible that Fyn in T cells (known as Fyn-T) might also be induced similarly thus resulting in the phosphorylation of LAT. Experiments are currently underway in the laboratory to knock down Fyn using morpholino anti sense oligos, to determine if ethanol can still induce tyrosine phosphorylation of LAT.

As a final indicator of T cell activation we showed that ethanol triggers both acute and prolonged calcium increases in T cells. We found that ethanol elevated the intracellular calcium $[Ca^{2+}]_i$ levels in a dose dependant manner, although the magnitude of $[Ca^{2+}]_i$ peaks in response to ethanol were generally smaller than those due to anti-TcR stimulation. However, low concentrations of antibody (2—8ng/ml), that gave calcium increases similar to ethanol were also able to suppress T cell responses.

Ethanol mediated spikes in $[Ca^{2+}]_i$ were not observed when cells were stimulated with ethanol in calcium free media suggesting that they arise due to the influx of calcium from outside the cell. From imaging data, we find that $[Ca^{2+}]_i$ levels in individual cells tend to oscillate when treated with ethanol. When cells were stimulated with ethanol in calcium free media, the oscillations were reduced and calcium levels did not rise above resting levels. This implicated the CRAC channels and their involvement was confirmed when Jurkat cells pretreated with 2-APB a CRAC channel inhibitor did not show any $[Ca^{2+}]_i$ elevation in response to stimulation with ethanol (Figure 4.4).

Acute exposure to ethanol leads to a spike in $[Ca^{2+}]_i$ and also modulates $[Ca^{2+}]_i$ elevations in response to subsequent antibody stimulation. Treatment with higher concentrations of ethanol leads to lower $[Ca^{2+}]_i$ elevation in response to stimulation with antibody. When cells are treated chronically (96 hours) with ethanol, $[Ca^{2+}]_i$ peaks in response to antibody is greatly reduced. Moreover the basal $[Ca^{2+}]_i$ level is elevated in

these cells. This is similar to what is seen with cells incubated in antibody for the same period of time. The suppression of the calcium response to antibody was reversible and cells started to show an increased response to antibody stimulation within 2 hours of the removal of ethanol. 24 hours after the removal of ethanol, the cells were observed to show a nearly normal response to antibody stimulation and also had returned to the $[Ca^{2+}]_i$ baseline seen in unstimulated cells.

At present, the data showing that ethanol induces unresponsiveness in Jurkat cells is clear but the mechanism for shutting of the signaling response in not. One possible reason for rendering T cells unresponsive is by triggering apoptosis and this has been reported [94, 148]. However in our studies, we did not uncover evidence of elevated cell death in cells cultured in ethanol.

Another possible mechanism for rendering T cell non responsive is anergy. Several studies have reported that sustained $[Ca^{2+}]_i$ elevation leads to the activation and translocation of NFAT into the nucleus[53]. In the absence of other transcription factors such as NFκB and AP-1, NFAT activates genes that inhibit the activation of T cells[54, 68]. Further more ethanol exposure has been linked to suppressed NFκB activation in monocytes [161, 162]. In cells chronically treated with ethanol, we saw that NFAT but not NFκB had translocated to the nucleus. T cell anergy is mediated by NFAT through the transcription of E3 ligases that target PKC-θ and PLC-γ for destruction[152, 153]. Immunoblotting lysates from Jurkat cells chronically treated with ethanol, we did not find evidence of PLC-γ degradation. At present, an effect of NFAT on LAT has not been reported but there are reports of decreased LAT phosphorylation in anergic cells[163, 164]. A recent study suggests that decreased LAT phosphorylation in anergic T cells may be due to defective recruitment of LAT to the immune synapse as well as defective

localization to the lipid rafts [165]. It would therefore be interesting to see if chronic exposure to ethanol has similar effects on LAT localization upon stimulation.

It has been reported that anergic T cells show increased Fyn activity and that this increased Fyn activity requires elevated $[Ca^{2+}]_i$ levels [166]. Moreover, in anergic cells, Fyn has also been found to be increasingly associated with Csk binding protein (Cbp or PAG)[167]. This association is reported to enhance TcR triggered calcium fluxes but is also reported to promote anergy. Interestingly, the C terminal Src kinase (Csk) is a cytoplasmic tyrosine kinase that serves as an essential and common regulator of the Src family of tyrosine kinases [168]. Csk is activated through phosphorylation by cyclic AMP dependant protein kinase (PKA). PKA as its name implies is activated by elevation of cAMP levels in T cells. Csk activation has been shown to suppress T cell activation. Moreover it has been reported that increasing cAMP levels in T cells increases tolerance[169].

While many questions still remain, the current data presents a plausible link between ethanol and immunosuppression. The data actually suggest two alternative possibilities. One possibility is that ethanol acts through LAT independent of calcium to cause immunosuppression and the other is that ethanol acts through calcium. Ethanol treatment closely parallels many of the events seen when T cells are treated with anti-TcR Ig including the prolonged elevation of calcium and translocation of NFAT to the nucleus. Since elevation of calcium using ionophores will itself render T cells unresponsive it seems plausible that prolonged elevation of calcium by ethanol would also have this effect. More experiments also need to be done to see what effect prolonged exposure to ethanol has on LAT and what correlation this has for the ability of ethanol to induce immunosuppression.

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